INVESTIGATING FUNCTIONS OF ERp29 IN MESENCHYMAL TO EPITHELIAL TRANSITION (MET) AND EPITHELIAL PLASTICITY IN BREAST CANCER CELLS

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Summary

Endoplasmic Reticulum protein-29 (ERp29) is a chaperone protein that functions in the unfolding and escort of secretory proteins. Like other reticuloplasmins, ERp29 is believed to be involved in carcinogenesis. In breast cancer, expression of ERp29 is downregulated and there exists a negative association between level of ERp29 and breast cancer stage/grade. To elucidate the role of ERp29 in breast cancer progression, aggressive breast cancer cells - MDA-MB231 and BT549 - were stably transfected with ERp29-expressing vectors. Upon isolation of single stable clones, morphological change from a spindle-like fibroblastic to a typical cobble-stone-like epithelial phenotype was observed in both ERp29-overexpressing MDA-MB231 and BT549 clones. This phenomenon is reminiscence of mesenchymal to epithelial transition (MET).

In malignancy, epithelial to mesenchymal transition (EMT) is believed to facilitate metastasis by medicating cells' escape from primary tumors. Its reverse, MET, has been considered both as counteract of EMT, thus preventing metastasis, as well as a mechanism employed by escaped cells to establish metastatic tumors at secondary sites, thus supporting metastasis. EMT/MET is characterized by morphological, molecular or behavioral changes in cells. In addition to the morphological change mentioned above, overexpression of ERp29 in MDA-MB231 cells induced behavioral changes typified by decrease in expression of mesenchymal cell markers (vimentin and fibronectin) and increase in expression of epithelial cell markers (E-cadherin,

β-catenin, and cytokeratin-19). These changes were believed to be brought upon downregulation of E-cadherin repressors (SNAI1, SNAI2, ZEB2, and Twist). Furthermore, ERp29-overexpressing MDA-MB231 clones exhibited lower migration and invasion capacity, indication of behavioral MET. In contrast, overexpression of ERp29 in BT549 cells only reduced the expression of fibronectin without changes in other markers and transcriptional repressors, as well as in cells' behavior.

Further investigation into the morphologic MET revealed that the morphological alterations observed in both cell lines were characterized by rearrangement of actin cytoskeleton, from stress fiber to cortical actin formation. In addition, mechanistic studies demonstrated that the levels of tight junction protein, ZO-1, and apical-basal polarity proteins, Par3 and Scribble, were markedly increased by ERp29 and mainly localized at the membrane to enhance cell-cell contact and polarization. However, other polarity proteins, including CDC42, Par6 and aPKC, did not seem to be involved in the ERp29-induced epithelial morphogenesis.

These findings demonstrated a novel function and mechanism of ERp29 in regulating epithelial plasticity. Though the consequences varied between cell lines (complete MET in MDA-MB231 cells and incomplete MET in BT549 cells), several common features were observed upon ERp29 overexpression; including rearrangement of actin cytoskeleton, regulation of cell-cell junctions, as well as cell polarization. Taken together, overexpression of ERp29 could reprogram aggressive breast cancer cells to induce MET and thus regulate metastasis.

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Conference abstract

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List of Abbreviations

aPKC	Atypical protein kinase C
ATP	Adenosine triphosphate
bHLH	Basic helix-loop-helix
BiP	Binding protein
BSA	Bovine serum albumin
CCKN2B	Cyclin-dependent kinase inhibitor 2B
CK19	Cytokeratin-19
CLD	Cytoplasmic lipid droplets
DAPI	4’,6-diamidino- 2-phenylindole
DMEM	Dulbecco’s modified eagle medium
Dlg	Discs large
DNA	Deoxyribonucleic acid
cDNA	Complementary DNA
pcDNA	Plasmid control DNA
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial to mesenchymal transition
ER	Endoplasmic reticulum
ERK	Extracellular receptor kinase
ERp29	Endoplasmic reticulum protein-29
FBS	Fetal bovine serum
HMEC	Human mammary epithelial cell
HRP	Horseradish peroxidase
Id	Inhibitor of differentiation
IgG	Immunoglobulin G
JAM1	Junctional adhesion molecule-1
JNK	Jun N-terminal kinase
Lgl	Lethal giant larvae
MAPK	Mitogen-activated protein kinase
MDCK	Madin-Darby Canine Kidney
MET	Mesenchymal to epithelial transition
MLC	Myosin light chain
pMLC	Phosphorylated myosin light chain
MMP	Matrix metalloproteinase
mTOR	Mammalian target of rapamycin
NF-κB	Nuclear factor kappa beta
PAK1	p21-activated kinase-1
PALS1	Protein associated lin seven-1

Par	Partitioning defective
PATJ1	PALS1-associated tight junction protein
PBS	Phosphate-buffered saline
PBST	PBS with tween-20
PCR	Polymerase chain reaction
RT-PCR	Reverse transcription polymerase chain reaction
PDGFR	Platelet derived growth factor receptor
PDI	Protein disulfide isomerase
PI	Propidium iodide
PI3K	Phosphoinositide-3- kinase
PVDF	Polyvinylidene Fluoride
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
mRNA	Messenger RNA
ROCK	Rho-associated coiled-coil containing protein kinase
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SIP1	Smad interacting protein-1
Sp	Specificity protein
TCF3	Transcription factor-3
TCF/LEF	T-cell factor/lymphoid enhancer factor
TGFβ	Transforming growth factor-β
TβR	Transforming growth factor-β receptor
UL35	Unidentified liver spot-35
UPR	unfolded protein response
USF	Upstream transcription factor
XBP	X-box binding protein
ZEB	Zinc finger E-box binding homeobox
ZO	Zonula occludens

Chapter 1: Introduction

1.1 Breast cancer

1.1.1 Incidence of breast cancer

Each year cancer is claiming millions of lives and affecting millions more. In developed country it is the leading cause of death and is the second in developing countries (reviewed in Jemal *et al.* 2011). GLOBOCAN 2008 worldwide statistics estimated 12.7 million cancer cases and 7.6 million cancer deaths in 2008 alone. Economically, in United States of America alone, The National Institute of Health estimated overall costs of cancer in 2010 at $263.8 billion which include medical, morbidity, and indirect mortality costs. This burden of cancer continues to increase as a result of growing and aging population as well as adoption of cancer-associated lifestyle.

Globally breast cancer is the most frequently diagnosed cancer in females (Jemal *et al.* 2011) (Table 1-1). It is predicted to account for 23% (1.38 million) of the total new cancer cases in 2008. Improvement in early detection is answerable for this increase of breast cancer incidence rate which has continually risen in the past 25 years. The same reason, together with better treatments, helps in the decline of breast-cancer related deaths. Despite all this, breast cancer is still the leading cause of cancer death in women (Jemal *et al.* 2011) with estimated 14% (458,400) of total cancer deaths in 2008, 90% of which is caused by metastatic breast cancer.

In Singapore, breast cancer is also one of the biggest cancer burdens. GLOBOCAN 2002 worldwide statistics ranked Singapore as having the highest incidence and mortality of breast cancer in Asia. In addition, Singapore Cancer Registry reported that breast cancer is the top cancer type affecting females, as well as one with the most deaths. This phenomenon will likely still occur in the future as since 1960s, the incidence rate has progress upwardly.

Estimated cancer cases affecting females worldwide			
Type	**Ranking**	**No.of cases**	**% of all cancers**
Breast	1	1384155	22.9
Cervix Uteri	2	530232	8.8
Colorectum	3	571204	9.4
Lungs	4	515999	8.5
Stomach	5	348571	5.8
Corpus Uteri	6	288387	4.8
Liver	7	226312	3.7
Ovary	8	224747	3.7
Thyroid	9	163968	2.7
Leukaemia	10	154978	2.6

Table 1-1 Predicted top ten most frequent cancers affecting women worldwide in 2008. Adapted from *J. Ferlay, F. Bray, P. Pisani and D.M. Parkin. GLOBOCAN 2008. Cancer Incidence, Mortality and Prevalence Worldwide. IARC Cancer Base No. 10. Lyon, France. International Agency for Research on Cancer. Year. Available at: http://globocan.iarc.fr/. Last accessed 05/04/2011.*

1.1.2 Classifications of breast cancer

Vast portion of human cancers (~90%) are carcinomas, *i.e*. cancers that arise in cells derived from epithelial origins (Elenbaas *et al.* 2001). Likewise, majority of breast cancers also originate from epithelia. Based on the starting site, breast carcinomas can be classified into two groups, lobular carcinoma that starts form the milk-generating glands (lobules) and ductal carcinoma which generates from ducts/tubes that carry milk from lobules to the nipples. In rare cases, breast cancer can also arise from other

areas and other cell types of the breast. Based on the invasiveness, these groups can be further classified into noninvasive (in situ) or invasive (infiltrating) carcinoma. The later covers cancers that invade their surrounding tissues as well as those that have metastasized to secondary sites. The most common form of invasive breast cancer is ductal invasive carcinoma which accounts for 75% of total cases, followed by lobular invasive carcinoma (10-15% of total cases) (reviewed in Vincent-Salomon *et al.*, 2003). Other rare types such as metaplastic carcinoma, mucinous carcinoma, as well as cribriform carcinoma, have also been described. Each of these invasive breast cancer types accounts for less than 5% of total cases. Common sites for breast cancer to spread are bone, lung, and liver (Hasebe *et al.*, 2008) and recently breast cancer metastasis to the stomach has been reported (Eo, 2008).

Invasive carcinoma is of great interest because, as mentioned earlier, it is believed to be responsible for 90% of cancer deaths (Fidler, 2002). Furthermore at the time of diagnosis, at least half of patients present clinically detectable metastasis (DeVita, *et al.*, 1975). In breast cancer, the 98% 5-year survival rate in noninvasive cancer drops if the cancer has spread (American Cancer Society. *Cancer Facts & Figures 2010*. Atlanta: American Cancer Society; 2010). The estimated survival rate falls to 84% if it only invades nearby tissues and 23% if it has metastasized to distant lymph nodes and organs.

The development of metastasis, including that of breast carcinomas, comprises of series of events. First cells must acquire migration and invasion capacity to escape from the primary tumor, penetrate the local stroma, and intravasate into the

bloodstream. At secondary sites cells must then extravasate where they can remain solitary (micrometastasis) or expand to form metastatic carcinoma. It is believed that the developmental program EMT is reactivated to facilitate the escape of cancer cells from primary sites and MET to facilitate metastasis formation at secondary sites.

1.2 EMT and MET

Since more than a century ago, the existence of the two main cell types, epithelium and mesenchyme, has been recognized. Epithelial cells are polarized in such a way that the top and bottom can be visually defined as apical and basal domain respectively (apical-basal polarization). The filamentous actin is also polarized with circumferential arrangement. Neighbouring epithelial cells are connected laterally to each other through cell-cell junctions which include adherens junctions, tight junctions, gap junctions, and desmosomes. Due to these intercellular links, individual cell movement is inhibited. Instead, epithelial cells must migrate as a group. On the other hand, mesenchymal cells do not establish stable cell-cell contacts which allows for increased individual migratory capacity. The actin filaments consist of network of interacting fibers. In addition, compared to epithelia, mesenchymes have more elongated shape and front-to-back leading edge polarity (reviewed in De Wever *et al.*, 2008). Although fairly rigid, epithelial cells are known to possess the ability to form mesenchymal cells, a process termed epithelial to mesenchymal transition (EMT) (Figure 1-1). Likewise, mesenhcymes also have the capacity to transform to epithelia, namely epithelial-mesenchymal reverse transition (EMrT) or mesenchymal to epithelial transition (MET) (Lillie, 1908).

EMT is a multistep process characterized by the loss of cell-cell junctions and reorganization of the cytoskeleton, which together result in the loss of apical-basal

cell polarity and the acquisition of spindle-shaped morphology (Huber *et al.*, 2005). This is associated with decrease in epithelial markers and increase in mesenchymal markers as well as gain of invasion and migration capacity. Accordingly, there are 3 aspects associated with EMT/MET; morphological, molecular, and behavioral alterations. Each of these elements will be explored further in subsequent sections.

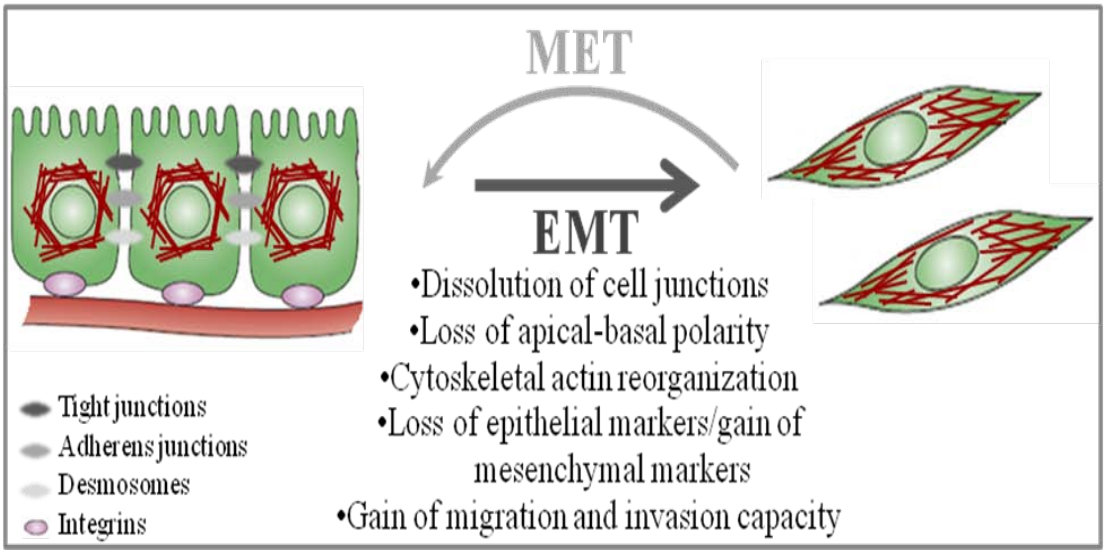


Figure 1-1 Illustration of EMT and its reversion MET. In the process of EMT, epithelial cells dissolve their cell-cell junctions, lose apical-basal polarity, and acquire mesenchymal properties characterized by stress fiber formation as well as increased migration and invasion capacity. During MET, mesencymal cells gain characteristics of epithelial cells.

EMT is found to be indispensible for normal development of multicellular organisms. During embryonic development, EMT is crucial for the formation of three-layered embryo through gastrulation (reviewed in Thiery *et al.*, 2009). It is also essential for various organ formations such as heart, musculoskeletal system, craniofacial structure, and peripheral nervous system. In adult, this process can be reactivated during wound healing as well as in pathological conditions such as organ fibrosis and carcinogenesis. The involvement of EMT in cancer progression was first suggested almost a decade ago (Thiery, 2002). Since then, it has been observed in variety of cancers such as ovarian, colon, oesophageal, as well as breast cancer (reviewed in Micalizzi *et al.*, 2010). Although sometimes debated (Tarin *et al.*, 2005), EMT is widely accepted as

one of the key mechanisms that facilitates metastasis by enabling epithelial-derived cancer cells to adopt a migratory and invasive phenotype and promoting escape from the primary sites (Thiery, 2002).

Of equal importance, MET is also heavily involved in embryonic development, where it alternates with EMT during the formation of heart and somite (reviewed in Chaffer *et al.*, 2007). In addition, MET is also known to be vital for kidney ontogenesis. In carcinogenesis, while EMT is believed to transform epithelial cells to more motile appearance, MET has been explored to explain the histopathological similarities between primary and metastatic tumors. It is hypothesized that MET occurs at secondary sites where sticky epithelial cells are able to extravasate from the bloodstream and form secondary metastasis (Thiery, 2002). This theory, however, remains highly controversial. Several studies have disregarded the idea that MET is an integral part of metastasis (Tsuji *et al.*, 2009; Graff *et al.*, 2000; Friedl *et al.*, 2003). Compared to EMT, MET is a relatively unknown subject as most studies have been devoted to unravel the mechanisms behind EMT. Therefore, in an effort to explain and illustrate in greater details, some of the discussions will be presented in the perspective of EMT.

1.2.1 Morphological changes in EMT/MET

Morphological change is the most obvious aspect and first indication of EMT/MET. During EMT the cuboidal epithelial cells are transformed into elongated spindle-like mesenchymal cells. This loss of epithelial morphology is contributed by the disruption of cell-cell junctions and apical-basal polarization as well as reorganization of actin cytoskeleton. Cell-cell junctions that are heavily studied in regards to EMT/MET are adherens and tight junctions. Despite their great importance in the formation and

maintenance of epithelial integrity, adherens junctions have been shown to be independent from morphological changes observed during EMT (Maeda *et al.*, 2005; Bhowmick *et al.*, 2001) and therefore will not be discussed in this section.

Tight junctions comprise of transmembrane proteins (occludin and claudins) whose cytoplasmic domains interact with several zona occludens (ZO) to form plaques that associate with the cytoskeleton (Tsukita *et al.*, 1997). These junctions serve as diffusion barrier for solutes and define the boundary between apical and basolateral membrane domains (Cereijido *et al.*, 1998). Dissociation of tight junctions is considered to be the first step of EMT and formation of tight junctions the completion of MET (Lee *et al.*, 2006). The formation of tight junctions is intimately linked to the proper polarization of cells which involves the participation of three polarity complexes; Par (Par3/Par6/aPKC) and Crumbs (Crumbs/PALS/PATJ) apical complexes which localized to the tight junctions and the Scribble (Scribble/Dlg/Lgl) basal complex (Dow *et al.*, 2007). These polarity modules often antagonize each other to mediate their proper positioning and functions (Bilder *et al.*, 2003). The loss of epithelial apical-basal polarization in EMT is attributed to the disruption of these proteins. Polarity modules, Par complex in particular, are also known to be essential for the assembly and maintenance of tight junctions (Izumi *et al.*, 1998; Joberty *et al.*, 2000). The associations between polarity proteins and cancer progression are complicated; they can act as tumor suppressors through their polarization activity or as oncoproteins when recruited as positive mediator for oncogenic pathways. Par3, for example, is diminished during carcinogenesis leading to loss of polarity in EMT

(Wang *et al.*, 2008) and Scribble is considered as tumor suppressors in various type of cancer (Dow *et al.*, 2007). In contrast, Par6 is genetically amplified in breast tumors (Nolan *et al.*, 2008) and activation or mislocalization of aPKC is considered as a factor that promotes tumor growth (Grifoni *et al.*, 2007).

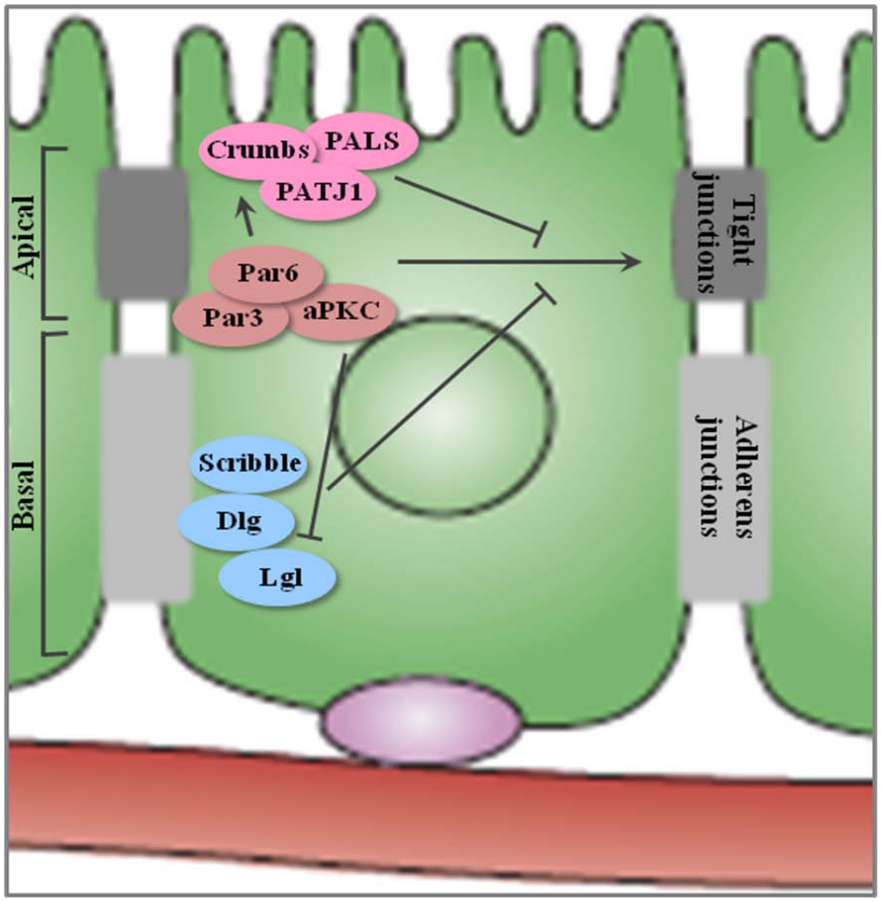


Figure 1-2 Diagram of polarity and junctional complexes. Par and Crumbs complexes are targeted to the tight junctions, in the apical region. Scribble complex is located in the basal region of epithelial cells. These complexes positively and negatively regulate each other for their proper localizations and functions.

The process of EMT also involves rearrangement of actin cytoskeleton from cortical actin to stress fiber formation. In MET, reorganization of the cytoskeleton is critical for the establishment of tight and adherens junctions as well as regulation of apical-basal polarity. One of the most well known regulators of cytoskeletal actin is the small GTPases family, in particular RhoA (Bishop *et al.*, 2000). Role of RhoA in

EMT/MET depends on its effectors. Activation of RhoA is known to induce EMT where through its downstream target Rho-associated coiled-coil containing protein kinase (ROCK), it regulates actin stress fiber formation and fibroblastoid morphology (Bhowmick *et al.*, 2001). On the other hand, the degradation of RhoA which results in the dissolution of tight junctions also leads to EMT (Ozdamar *et al.*, 2005). mDia, another effector of RhoA is believed to be responsible for this effect (Ozdamar *et al.*, 2005).

1.2.2 Molecular changes in EMT/MET

Molecular changes in EMT/MET include regulation of epithelial markers (E-cadherin, cytoplasmic β-catenin, cytokeratins) and mesenchymal markers (fibronectin, vimentin, nuclear β-catenin, N-cadherin). Among the markers, E-cadherin is considered the master regulator of EMT/MET where loss of its function and/or expression is heavily involved in EMT (Thiery, 2002). Indeed, during development loss of E-cadherin has been observed at EMT sites (Damjanov *et al.*, 1986; Tepass *et al.*, 1996); accordingly it is expressed at sites of MET during kidney ontogenesis (Kuure *et al.*, 2000). Furthermore, downregulation of E-cadherin expression is one of the most frequently reported characteristics of metastatic cancers (Birchmeier *et al.*, 1994), and restoration of E-cadherin in cancer cells leads to suppression of invasive and metastatic ability of cancer (Vleminckx *et al.*, 1991; Frixen *et al.*, 1991; Perl *et al.*, 1998). Loss of E-cadherin is particularly observed in invasive front of primary tumors; conversely its reexpression in metastatic tumors suggests that E-cadherin is involved in MET at secondary sites (Kowalski *et al.*, 2003; Yates *et al.*, 2007). In general, level of

E-cadherin in primary tumors is conversely related to cancer grade or patient survival (Birchmeier *et al.*, 1994; Hirohashi, 1998).

E-cadherin is a major component of adherens junctions in epithelial cells. It is a transmembrane glycoprotein whose extracellular domain mediates lateral cell-cell contacts by forming homotypic binding with E-cadherin of neighboring cells. Its cytoplasmic domain interacts with actin cytoskeleton via α-catenin and β-catenin. The loss of E-cadherin or its relocalization from cell membrane releases β-catenin which will translocate to nucleus where it functions in the Wnt pathway. In nucleus, β-catenin interacts with members of the T-cell factor/lymphoid enhancer factor (TCF/LEF) family of transcription factors and modulates expression of a large number of genes involved in cell proliferation, migration, invasion, and morphogenesis which further mediates the progress of EMT (Clevers, 2006). Loss of E-cadherin is also associated with the induction of N-cadherin, a phenomenon called cadherin switch (Christofori, 2003). While cadherin switch is important for the molecular as well as behavioral alteration of EMT, it has been shown to have little or no effect on the morphological aspect. In fact, switching from E-cadherin to N-cadherin protein expression occurs only after EMT-like morphological changes become apparent (Lindley *et al.*, 2010).

The loss of E-cadherin expression and/or function can be achieved by transcriptional repression, promoter hypermethylation, or gene mutation. The first mechanism has emerged as one of the critical steps driving EMT. Several transcription factors are known to regulate E-cadherin transcriptionally and they have been shown to regulate

EMT both in normal physiological and pathological conditions. The identified Snail (SNAI1 and SNAI2), ZEB (ZEB1 and ZEB2/SIP1), and the basic helix-loop-helix (TCF3/E2A, Twist, Id2, Id3) families bind to consensus E-boxes on the E-cadherin promoter thus repressing its expression (Huber *et al.,* 2005; Peinado *et al.,* 2007). In addition, these transcription factors are also known to regulate each other as well as the expression of other EMT-related genes, including tight junction components, desmosomes, and matrix metalloproteinases, as well as polarity proteins (reviewed in Xu *et al.*, 2009; Moreno-Bueno *et al.,* 2008). They are also responsible for the change of intermediate filaments from epithelial-cytokeratins to mesenchymal-vimentin. Thus the transcription factors are responsible for the programming of cells toward mesenchymal state.

These transcription factors are closely associated with EMT/MET in both physiological and pathological conditions. In embryonic development, SNAI1 and SNAI2 are known to control gastrulation and neural-crest EMT in different species (Thiery, 2003). In cancer progression, SNAIl is associated with a diffuse tumor type in gastric carcinoma and ZEB2 is associated with intestinal-type gastric carcinoma (Hajra *et al.*, 2002; Blanco *et al.*, 2002; Rosivatz *et al.*, 2002). Knockdown of Twist in cancer cells prevented metastasis and its overexpression results in repression of E-cadherin as well as complete EMT (Yang *et al.,* 2004). Generally in the process of EMT, the changes in the transcriptional program described in this section are complemented by non-transcriptional changes (described earlier) that help define the changes in cytoskeletal organization and cell shape.

1.2.3 Behavioral changes in EMT/MET

Migration is a fundamental ability of mesenchymes by which they can reach their destination to carry out their particular functions. Due to the intricate cell-cell junctions, this characteristic is absent in epithelial cells. Therefore, on top of the morphology and molecular alterations, epithelial cells undergoing EMT may also gain migratory as well as invasion capacity. During EMT, increased level of extracellular components including collagens and fibronectin is observed. These proteins stimulate integrin signaling and induce the formation of focal adhesion which facilitate migration (Imamichi *et al.*, 2007; Zhao *et al.*, 2009). The formation of focal adhesion is also induced by focal adhesion kinase that is activated upon downregulation of E-cadherin (Frame *et al.*, 2008). Upregulation of N-cadherin, which is associated with loss of E-cadherin, also promotes cell migration as cell-cell contacts formed by N-cadherin are much weaker than those of E-cadherin (Cavallaro *et al.*, 2002; Hsu *et al.*, 1996).

Increased migration capacity is often translated into invasive behavior as cancer cells express and activate their extracellular proteases, such as matrix metalloproteases (MMPs), allowing them to degrade extracellular matrix proteins and escape their surrounding (Moustakas *et al.*, 2007). The accumulation of nuclear β-catenin contributes to this phenomenon since together with TCF/LEF transcription factor, it regulates the expression of MMPs. In addition, Snail and ZEB transcription factor families are also known to induce the expression of these proteases (reviewed in Xu *et al.*, 2009).

Studies have shown that EMT-cells which have gained motility are responsible for the degradation of surrounding matrix to enable invasion and are often found in the invasive front of primary tumors (Tsuji *et al.*, 2009). On the other hand, induction of MET has been reported to suppress tumor growth and metastasis in vivo (Chiu *et al.*, 2009). It is worth noting that despite the indisputable involvement of EMT, it is not the only mechanism by which cells gain migration and invasion capacity in carcinogenesis. There exist other scenarios where cancer cells become more migratory and invasive without the occurrence of EMT (Pinkas *et al.*, 2002; Wicki *et al.*, 2006).

1.2.4 EMT/MET in breast cancer and its clinical implications

Like in other cancer types, the occurrence and therefore significance of EMT/MET in breast cancer progression had been debated. The main reason is intricacy in following EMT/MET in time and space, especially in the heterogeneous environment of breast carcinoma. Furthermore, due to its transient nature (where EMT is followed by MET) pathologists are finding it difficult to observe cells undergoing EMT in clinical samples. However, indirect evidences of the involvement of EMT in breast cancer progression have accumulated over the years.

The existence of isolated single cells in ductal invasive carcinoma suggests the occurrence of EMT from this epithelial-origin cancer; as well as the intermixed of epithelial and non-epithelial cells in metaplastic carcinoma (reviewed in Vincent-Salomon *et al.*, 2003). Furthermore, the phenotype of breast cancer micrometastases in lymph nodes and in the bone marrow also indicates that EMT occurs within the primary tumors (Braun *et al.*, 1999). Loss of expression and/or

functions of epithelial marker, E-cadherin, is frequently observed in breast carcinoma (Berx *et al.*, 1998; Cheng *et al.*, 2001). On the other hand, the emergence of mesenchymal marker, vimentin, in epithelial cells of breast tumors correlates with a shorter post-operative survival of patients. The various repressors of E-cadherin also associates with breast cancer progression; their overexpression is positively related to tumor aggressiveness and recurrence, poor prognosis as well as survival (reviewed in Peinado *et al.*, 2007). Microarray-based study revealed that the expression of EMT markers preferentially occurs in basal-like breast tumors which are related to their poor prognosis and distant metastasis (Sarrió *et al.*, 2008).

Direct evidence of EMT in breast cancer has recently been presented (Trimboli *et al.*, 2008). In the study, epithelial cells were tracked and found to give rise to stromal fibroblasts upon *in vivo* tumor induction by oncogene *myc*. These cells lacked epithelial-cytokeratins and E-cadherin while expressing mesenchymal-vimentin and fibronectin.

Considering its involvement in cancer progression, in particular metastasis formation, targeting EMT may have significant therapeutic effects in preventing invasion as well as metastasis. Several marketed drugs such as PDGFR inhibitor imatinib (Gleevec), HER2 inhibitor trastuzumab (Herceptin), and EGFR inhibitor gefitinib (Iressa) have been shown to inhibit EMT in breast cancer progressions as well as cancer patients (reviewed in Huber *et al.*, 2005). In contrast, treatments such as chronic chemotherapy with oxaliplatin and ionizing radiations have been shown to induce EMT (Yang *et al.*, 2006; Jung *et al.*, 2007). Another reason for the urgency of EMT inhibition rises from

the fact that EMT confers drug resistance. Lung cancer cells that have undergone EMT are insensitive to the growth inhibitory effects of EGFR kinase inhibition (erotinib) as well as other EGFR inhibitors such as gefitinib and cetuximab (Thomson *et al.*, 2005; Frederick *et al.*, 2007; Fuchs *et al.*, 2007). In addition, targeting EMT may also prevent recurrence as it has been associated with residual breast cancer cells that survive following conventional chemotherapy (Creighton *et al.*, 2009). Therefore, understanding EMT/MET in breast cancer is of great importance as it may not only halt cancer progression to a metastatic state but also its recurrence.

1.3 ERp29

1.3.1 Structure and distribution

ERp29 is a reticuloplasmin, protein that resides in the endoplasmic reticulum (ER) lumen. Hubbard's group was the first to clone this protein from rat enamel cells (Demmer *et al.*, 1997). Further studies unified various ERp29-homologues, unidentified liver spot 35 (UL35) and ERp31, as products of a single gene and correlated human ERp29 with cognate cDNA previously name ERp28 and ERp31 (Hubbard *et al.*, 2000a). The same group also isolated human ERp29 from liver and revealed striking homologies both in sequence and physical properties of the protein from both sources (Hubbard *et al.*, 2000a). In fact, ERp29 is highly conserved among mammals, with homolog Windbeutel found in organism as primitive as Drosophila (Hubbard *et al.*, 2000b). ERp29 is ubiquitously expressed in most if not all of fetal and adult mammalian cells and tissues with high level of expression examined in

secretory tissues such as adrenal, mammary, enamel, prostate, thyroid, and liver (Mkrtchian *et al.*, 1998b; Hubbard *et al.*, 2000a; Liepinsh *et al.*, 2001; Sargsyan *et al.*, 2002b). These observations, together with the characteristics of its promoter (GC rich, absence of TATA box, and presence on multiple transcription start-sites) indicate that ERp29 is a constitutively expressed housekeeping gene with a function of general importance (Sargsyan *et al.*, 2002a).

ERp29 gene is mapped to chromosome 12q24.13 and contains three small exons separated by one small and one large introns (Sargsyan *et al.*, 2002a). The predicted GC and E box elements within the promoter have been shown to interact with Sp1/Sp3 and USF1/USF2 transcription factors respectively. ERp29 gene encodes for 261-residue protein that is of 25.6kDa in size (Demmer *et al.*, 1997). Secondary structure analysis indicates that ERp29 is generally hydrophilic with a strong hydrophobic N-terminus containing ER-targeting peptide which will be cleaved in mature protein (Mkrtchian *et al.*, 1998b). The C-terminus contains KEEL motif, a variant of ER-retention motif that is recognized by specific receptor that continually retrieves the protein from later compartment of secretory pathways and returns them to the ER (Mkrtchian *et al.*, 1998b).

Tertiary structure of ERp29 is characterized by two domains connected by a flexible linker (residue 149-159). The N-terminal domain has a typical α/β thioredoxin fold that is similar to that of protein disulfide isomerase (PDI) but without the double-cysteine motif important for disulfide-bond formation (Barak *et al.*, 2009). The C-terminal domain shows high similarities to Windbeutel (Lippert *et al.*, 2007;

Barak *et al.*, 2009) and contains a novel helical fold (Liepinsh *et al.*, 2001). In fact, the C-terminal domain of ERp29 can be exchanged with that of Windbeutel to process Pipe, a Windbeutel substrate (Nilson *et al.*, 1998). Several conserved residues in helix 8 (Glu222, Arg225, Lys228, and Leu 229) and helix 9 (Leu242) have been shown as the substrate binding site (Barak *et al.*, 2009; Lippert *et al.*, 2007). The Cys125 and Cys157 residues play a key structural role in stability of the C-terminal domain (Hermann *et al.*, 2005; Baryshev *et al.*, 2006). The first is also important for the hydrophobicity of interdomain linker.

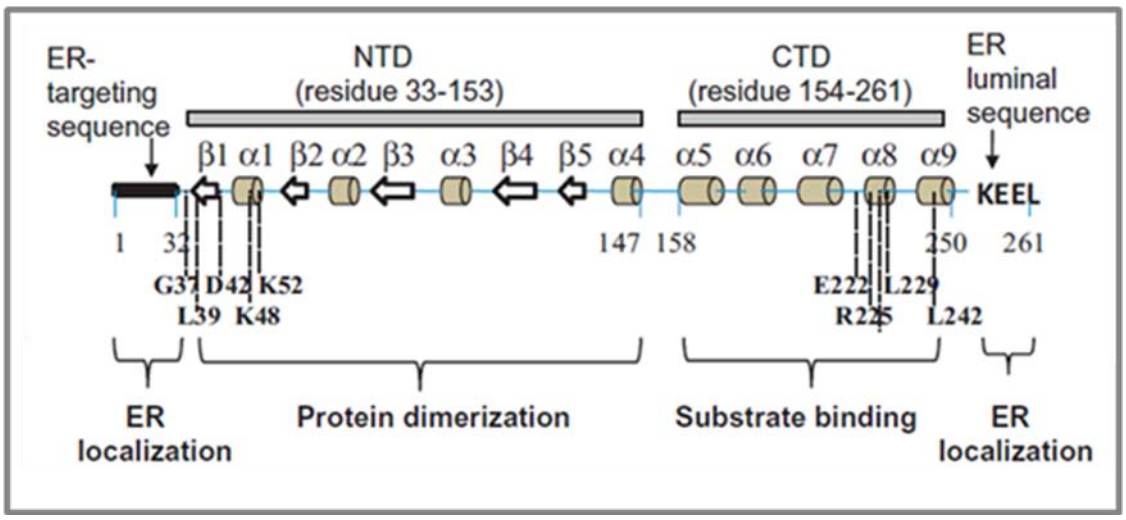


Figure 1-3 Secondary structure of ERp29. The structure reveals an ER-targeting sequence at N-terminus and a variant of ER-retention motif at C-terminus. *Figure was sourced with permission from Zhang D and Richardson DR: Endoplasmic reticulum protein 29 (ERp29): An emerging role in cancer. Int J Biochem Cell Biol. 43: 33-36.*

Size exclusion chromatography, cross-linking, and dynamic light scattering studies suggest oligomerization of ERp29 (Mkrtchian *et al.*, 1998a; Ferrari *et al.*, 1998). Its dimerization in particular is of importance and essential for its diverse functions. ERp29 mutant that lose the ability to dimerize efficiently is unable to mediate Polyomavirus infection and thyroglobulin (Tg) secretion (Rainey-Barger *et al.*, 2007).

The N-terminal domain exclusively mediates and is essential for the dimerization (Liepinsh *et al.*, 2001). Mutagenesis study further revealed that residues Gly37, Leu 39, Asp42, Lys48, and Lys52 contribute to the dimerization (Rainey-Barger *et al.*, 2007; Lippert *et al.*, 2007).

Consistent with the presence of ER-retention motif, ERp29 has been shown to localize in the luminal part of ER by biochemical and morphological analysis (Mkrtchian *et al.*, 1998b). This localization however is not exclusive, as together with its substrate Tg ERp29 is co-secreted (Sargsyan *et al.*, 2002b). Furthermore, ERp29 was also identified in cytoplasmic lipid droplets (CLD) produced during lactation (Wu *et al.*, 2000). Tissue staining revealed presence of ERp29 in nuclei in tumor and control cells (Cheretis *et al.*, 2006). The significance of this localization is not yet clear.

Compare to most ER proteins, ERp29 is unique as it does not have the expected post-translational modifications and ATP-dependent properties (Ferrari *et al.*, 1998). In addition, calcium binding motif and ER-stress response element that can be found in other reticuloplasmins are absent in ERp29 (Demmer *et al.*, 1997). Therefore, despite structural similarities with other proteins, such as PDIs and Windbeutel, ERp29 may have different, while complementary, functions to other PDI-like proteins within ER system.

1.3.2 Functions

In eukaryotic cells, ER functions in the production of secretory proteins and calcium regulations (Brodsky *et al.*, 1997; Dorner *et al.*, 1990). Serving these vital roles are reticuloplasmins such as PDIs, Binding Protein (BiP), calreticulin, and endoplasmin. These proteins have overlapping tasks such as protein-folding assistants and calcium buffers. Lack of calcium binding and double-cysteine motifs precludes ERp29 as calcium buffer and disulphide isomerase, leaving it as a possible protein-folding assistant. A distinct role of ERp29 in secretory events is implied by its high expression in secretory tissues (Hubbard *et al.*, 2000b; Shnyder *et al.*, 2000), inducibility under ER-stress condition (Mkrtchian *et al.*, 1998b), PDI-like cellular expression profile and BiP-like predominant location in the rough ER (Shnyder *et al.*, 2000), as well as colocalization with other ER-chaperones (Mkrtchian *et al.*, 1998b). Furthermore, ERp29 is only found in multicellular organism where protein export function is extensively developed (Sargsyan *et al.*, 2002a).

ERp29 has been implicated in the production and/or secretion of various proteins including thyroglobulin, connexin 34, as well as soluble milk proteins (Baryshev *et al.*, 2006; Das *et al.*, 2009; Mkrtchian *et al.*, 2006). In addition it is known to regulate the ER membrane penetration during Polyomavirus infection, sperm maturation, as well as production of endomembrane proteins (Magnuson *et al.*, 2005; Ying *et al.*, 2010; MacLeod *et al.*, 2004).

ERp29 is also involved in the ER-stress response which triggers an unfolded protein response (UPR) characterized by transcriptional induction of genes that enhance

protein folding capacity and general translational attenuation to reduce protein load in the ER (Mkrtchian *et al.*, 1998b). Since ERp29 lacks the ER stress response element, ER-stress induced ERp29 is thought to be regulated via XBP1/IRE1 pathway (Mkrtchian *et al.*, 1998a; Bambang *et al.*, 2009a). X-binding protein-1 (XBP1) is a key regulator of UPR that works by binding to DNA element other than ER-stress response element. It has been reported that XBP1 and p38 negatively regulates ERp29 expression, while overexpression of ERp29 activates XBP1 (Bambang *et al.*, 2009a; Zhang *et al.*, 2010a).

1.3.3 ERp29 in cancer development

During carcinogenesis, physiological and/or pathological stimuli such as nutrient-depletion, oxidative stress, DNA-damage, calcium-deprivation, growth factors and oncogenic factors, have been shown to perturb ER homeostasis. Under these conditions, the unfolded/misfolded proteins accumulate, leading to ER stress and the activation of ER-specific signalling pathways (reviewed in Ron *et al.*, 2007). Therefore, it is not surprising that major reticuloplasmins such as BiP and PDI are established key players in cancer development. The first is an attractive target for cancer therapy due to its role in protein production and survival of cellular stress while the later is proposed as biomarker due to its broad overexpression (Ma *et al.*, 2004; Ma *et al.*, 1997). Consequently, efforts have been devoted to elucidate role of ERp29, a novel reticuloplasmin, in cancer development. However, conflicting results have emerged, implicating ERp29 as both oncogene and tumor suppressor. Table 1-2 summarizes the findings of Erps29's expression and role in carcinogenesis.

ERp29 as oncogene	ERp29 as tumor supressor
ERp29 is upregulated in epithelial tumors (mammary, salivary, bladder, prostate, ovary, kidney, skin) (Shnyder *et al.* , 2008).	ERp29 is downregulated in pancreatic cancer (Lu *et al.* , 2004).
Direct relationship of ERp29 and tumor prognosis in basal cell carcinoma of the skin and ovarian tumor (Cheretis *et al.* , 2006; Bengsston *et al.* , 2007).	Inverse relationship of ERp29 and tumor prognosis in lung and colon cancer (Shnyder *et al.* , 2008).
Overexpression of ERp29 in endometrial and breast cancer (Mkrtchian *et al.* , 2008).	ERp29 is downregulated in breast cancer samples and is inversely regulated with cancer grade/stage (Bambang *et al.* , 2009b).
Cancer cell lines SK-N-SH, A549, A375, MCF7, and Hela express ERp29 (Myung *et al.* , 2004).	Cancer cell lines Saos2, CaOv3, HCT116, HL60 and A673 do not express ERp29 (Myung *et al.* , 2004).
Dominant negative and silencing of ERp29 in MCF7 breast cancer cell line results in size-reduction of tumor xenografts (Mkrtchian *et al.* , 2008).	Overexpression of ERp29 in MDA-MB231 breast cancer cell line results in size-reduction of tumor xenografts (Bambang *et al.* , 2009b).

Table 1-2 Studies on the relationship of ERp29 and cancer development. The left column summarizes those that support the idea that ERp29 is oncoprotein, the right column suggests that ERp29 is a tumor suppressor.

1.4 Rationale of work

The ubiquitous and conserved expression of ERp29 suggests that it is imperative in basic cell functions. Thus, coupled with the importance of many reticuloplasmins in carcinogenesis, dysregulation of ERp29 might also contribute to tumor progression. Preliminary studies conducted were mainly focused on the associations of ERp29 expression level and status and/or stage of cancer which revealed that ERp29 is differentially expressed in various cancers. There was, however, lack of effort to unravel how ERp29 functions in carcinogenesis. This prompted initiation of current study where overexpression of ERp29 in breast cancer cells was employed to elucidate the role of ERp29 in breast cancer progression.

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Cell Lines

All cell lines used in this research project were purchased from the American Type Culture Collection (ATCC, VA, USA). The metastatic MDA-MB231 and BT439 breast cancer cell lines were grown in Dulbecco's modified eagle medium (DMEM, Sigma, USA) and RPMI-1640 (GIBCO®, USA) respectively, supplemented with 10% fetal bovine serum (FBS; GIBCO®, USA). The ERp29-transfected MDA-MB231 and BT549 clones, as well as the vector-transfected control cells, were maintained in their respective media with 1mg/ml or 2mg/ml of G418 (Invitrogen, Oregon, USA) respectively. All the cell lines were grown at 37°C in a humidified 5% CO_2 incubator.

2.1.2 Antibodies

Primary antibodies used in this research project along with the dilution for immunoblotting and immunofluorescence analysis are surmised in Table 2-1.

Target	Company	Dilution	
		Immunoblot	Immunofluorescence
aPKC	Cell Signaling Technology (MA, USA)	1:1000	1:200
β-actin	Sigma (MO, USA)	1:10000	-
β-catenin	Cell Signaling Technology (MA, USA)	1:1000	1:200
ERp29	(Acris, Hiddenhayse, Germany)	1:2500	1:500
E-cadherin	BD Biosciences PharMingen (CA, USA)	1:2500	1:500
Fibronectin	Cell Signaling Technology (MA, USA)	1:500	1:100
pMLC	Cell Signaling Technology (MA, USA)	1:1000	1:200
Par3	Novus Biological (CO, USA)	1:1000	1:200
Par6	Novus Biological (CO, USA)	1:1000	1:200
Scribble	Millipore (CA, USA)	1:500	1:100
SNAI1	Abnova (Taipei, Taiwan)	1:200	1:50
SNAI2	Abnova (Taipei, Taiwan)	1:200	1:50
TCF3	Abnova (Taipei, Taiwan)	1:1000	1:200
Twist	Cell Signaling Technology (MA, USA)	1:1000	1:200
Vimentin	Millipore (CA, USA)	1:5000	1:1000
ZEB1	Abnova (Taipei, Taiwan)	1:2500	1:500
ZEB2	Abnova (Taipei, Taiwan)	1:1000	1:200
ZO1	Cell Signaling Technology (MA, USA)	1:1000	1:200

Table 2-1 List of primary antibodies.

2.1.3 Primers

All the primers used were synthesized by 1st BASE (Singapore). Table 2-2 represents the sequence for each primer.

Gene	Primer sequence	
aPKC	Forward	5’- TACGGCCAGGAGATACAACC - 3’
	Reverse	5’- TCGGAGCTCCCAACAATATC - 3’
β-actin	Forward	5’-CCTTCCTGGGCATGGAGTCCTG-3’
	Reverse	5’-GGACAATGATCTTGATCTTC-3’
ERp29	Forward	5’-TCTCCTGGGCTTCCTGCTCCTCTC-3’
	Reverse	5’-TATTGCTCGGCCCACTTCTTCTGA -3'
Par3	Forward	5’- GCAGTGTTTGATGAGCAGGA - 3’
	Reverse	5’- TTGCTCGAAGGACTGAAGGT - 3’
Par6	Forward	5’- GTTGCCAACAGCCATAACCT - 3’
	Reverse	5’- CAGGTCACTGCTGTCATCGT - 3’
Scribble	Forward	5’- CGCAAGGACACACCTCACTA - 3’
	Reverse	5’- CCTCCTCCTGAGGACTACCC - 3’
SNAI1	Forward	5’- CTGCAGGACTCTAATCCAG - 3’
	Reverse	5’- CGAGAGACTCCGGTTCCTA-3’
SNAI2	Forward	5’-AGCGAACTGGACACACATAC-3’
	Reverse	5’-TCTAGACTGGGCATCGCAG-3'
TCF3	Forward	5’-GTCCCTGGAGGAGAAAGACC-3’
	Reverse	5’-CTGCTTTGGGATTCAGGTTC-3’
Twist	Forward	5’-GCAAGCTTAGAGATGATGCAGGACG-3’
	Reverse	5’-GACTCGAGGTGGGACGCGGACATGGA-3’
ZEB1	Forward	5'-CTGCTGGGAGGATGACAGA-3'
	Reverse	5'-ATCCTGCTTCATCTGCCTGA-3'
ZEB2	Forward	5'-AGTCCATGCGAACTGCCATCTGAT-3'
	Reverse	5'- CTGGACCATCTACAGAGGCTTGTA-3'
ZO-1	Forward	5’- TGGTGTCCTACCTAATTCAACTCA - 3’
	Reverse	5’- CGCCAGCTACAAATATTCCAACA - 3’

Table 2-2 List of primer sequence.

2.2 Methods

2.2.1 Construction of ERp29-expression vector

The full length cDNA of human ERp29 gene was amplified by PCR with the Platinum High Fidelity Taq DNA polymerase (Invitrogen, Oregon, USA) using the forward primer (5-ATATGAATTCATGGCTGCCGCTGTGC-3'with EcoRI site underlined) and the reverse primer (5'-TCAGGATCCCTACAGCTCCTCCTCTTT-3'with BamHI site underlined). The PCR product was digested with BamHI and EcoRI and then cloned into BamHI and EcoRI sites of pcDNA3.1 (+) vector (Invitrogen, Oregon, USA) to form expression vector pcDNA-ERp29. The authenticity of ERp29 gene sequence was confirmed by DNA sequencing (primers used are listed in Table 2-2).

2.2.2 Generation of ERp29-overexpressing single stable clones in MDA-MB231 and BT549 breast cancer cells

To create ERp29-overexpressing clones, ERp29-pcDNA3.1 vector obtained above was used to transfect MDA-MB231 and BT549 cells. The empty vector (pcDNA3.1) served as control. Cells were seeded in a 6-well plate to a confluency of 60-70%. For each well, 1μg of plasmid vector was diluted in the appropriate amount of Opti-MEM®I reduced serum medium (Invitrogen, Oregon, USA) and the cells were transfected using LipofectAMINE^{TM} 2000 (Invitrogen, CA, USA), according to the manufacturer's instructions. Two days after the transfection, selecting agent G418 was added to select for successful transfectants. For single clone generation, serial dilutions were performed to obtain single cells. Each colony produced from these

single cells was verified for their ERp29 expression using reverse-transcription PCR and immunoblot assay. Two ERp29-overexpressing clones for MDA-MB231 (clone B and E) and for BT549 (clone A and K) were used in subsequent experiments.

2.2.3 RNA extraction and reverse-transcription polymerase chain reaction (RT-PCR)

Total RNA from cultured cells was extracted using NucleoSpin® RNA II kit (Macherey-Nagel GmbH & Co. KG, Germany) according to the manufacturer's protocol. Briefly, $5x10^6$ cells were trypsinized, washed, pelleted and subsequently lysed in lysis buffer containing β-mercaptoethanol. The lysate was then filtered and passed through the provided NucleoSpin® RNA II Column for RNA binding followed by DNA digestion. Purified RNA was eluted and its concentration was determined using Nanodrop Spectrophotometer ND-1000 (Thermo Fisher Scientific, Lafayette, CO, USA).

0.5µg of purified RNA template was used to synthesize first strand cDNA using ImProm-II reverse transcriptase (Promega, WI, USA). This reverse transcription was performed at 42°C for 1h, followed by 70°C for 15min. The cDNA was then amplified by semi-quantitative PCR using respective specific primers (Table 2-2). This amplification was carried out as shown in Table 2-3 using Thermal Cycler GeneAmp®PCR System 9600 (Applied Biosystems, CA, USA).

Steps	Temperature (°C)	Time
1. Initializing	94	5min
2. Denaturation	94	30s
3. Annealing	According to sequence of primer	30s
4. Extension/elongation	72	30s
5. Final elongation	72	7min
6. Final hold	4	~

**Table 2-3 PCR amplification steps. ***30 cycles (Steps 2-4)

Amplified DNA was finally run on 1% DNA agarose gel (Seakem®LE Agarose, Cambrex Bio Science Rockland, Inc., Rockland, ME, USA) containing 0.1μg/ml Ethidium Bromide at 90V for 40 min. Fluorescence signal was captured with the MULTI GENIUS BioImaging System (Syngene, Frederick, MD, USA). The level of β-actin served as the loading control.

2.2.4 Protein extraction and immunoblot/western blot assay

To prepare total cell lysate, cells were trypsinized and washed once with phosphate-buffered saline (PBS, pH 7.4). The cells were then resuspended in cold RIPA buffer (1% Igepal, 1% sodium deoxycholate, 0.15M sodium chloride, 0.01M sodium phosphate, pH 7.2, and 2mM EDTA) supplemented with protease inhibitors (Roche Diagnostics, Indianapolis, IN) and phosphatase cocktail inhibitors I and II (1:100; Sigma-Aldrich, Steinheim, Germany), and kept on ice for 2hr to ensure total lysis. Cell lysate was then centrifuged at 4°C and the supernatant was collected. Protein concentration was determined using the Coomassie Plus Bradford assay (Pierce, Rockford, IL).

Depending on the size and relative expression of each protein tested, 15 to 75μg of total proteins was separated on 8-12% SDS-PAGE gels using Mini-PROTEAN 3

Electrophoresis Cells (Bio-Rad, Hercules, CA, USA) at 70V for 30min followed by 100V until protein of interest is separated well. The proteins were then transferred onto Hybond-P Polyvinylidene Fluoride (PVDF) membrane (GE Healthcare, Uppsala, Sweden) using the wet transfer apparatus (Bio-Rad, Hercules, CA, USA) at 110V for at least 1hr. The membrane was blocked with 5% non-fat milk (Santa Cruz Biotechnology, Inc., CA, USA) in Tris-buffered saline containing 0.1% Tween-20 (TBST) and incubated overnight with appropriate primary antibodies at 4ºC. Upon washing, secondary antibodies - HRP-conjugated goat anti mouse IgG (Molecular Probes, Invitrogen, Oregon, USA) at 1:5000 or HRP-conjugated goat anti rabbit IgG (ZYMED® Laboratories, Inc. San Francisco, CA, USA) at 1: 10,000 or HRP-conjugated rabbit anti goat IgG (Molecular Probes, Invitrogen, Oregon, USA) at 1:5000 - were applied for 1h at room temperature. The chemiluminescent signals were visualized using SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA) and captured with the MULTI GENIUS BioImaging System (Syngene, Frederick, MD, USA). Signal intensity was analyzed by the GeneTools software (Syngene, Frederick, MD, USA). The level of β-actin was used as the loading control.

2.2.5 Immunofluorescence and confocal microscopy

Cells were plated onto 12mm glass coverslips and incubated overnight at 37°C in a humidified 5% CO_2 incubator to allow attachment. The following day, cells were rinsed twice in PBS and fixed with 4% paraformaldehyde (Sigma-Aldrich, Steinheim, Germany) for 15mins and permeabilized with 0.1% Triton X-100 for 10mins. This

was followed with blocking in 3% BSA for 1hr at room temperature and overnight incubation in primary antibody at 4°C. Upon washing with PBS containing 0.02% Tween-20 (PBST), cells were incubated for 1hr at room temperature with anti-mouse Alexa Fluor® 488 goat anti-mouse IgG (1:1000; Invitrogen, CA, USA) or Alexa Fluor® 532 goat anti-rabbit IgG (1:1000; Invitrogen, CA, USA). To visualize actin filaments, cells were stained with rhodamine-phalloidin (1:500, Invitrogen, Oregon, USA) for 45min at 37ºC. After final washes with PBS, the coverslips were mounted using the antifade mounting fluid containing DAPI (Vector Laboratories, Inc, CA, USA) and the images were examined and captured using Olympus Fluoview FV500 confocal laser scanning microscope (Olympus, Japan). Raw images were analyzed using Olympus FV10-ASW 1.7 Viewer software (Olympus, Japan).

2.2.6 Cell proliferation assay

Cells ($1x10^3$ per well) were seeded in triplicate in 96-well culture plates and cultured for 4 days at 37°C in a humidified 5% CO_2 atmosphere. Viable cells were quantified at 24hr interval using CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (Promega, WI, USA). The absorbance at 492nm was measured using an Infinite F200 microplate reader (TECAN Austria GmbH, Grodig, Austria).

2.2.7 Cell cycle assay

Cells were starved for 24hr and released from starvation for another 24hr. Following that, cells were washed twice in ice-cold PBS and fixed in 75% ethanol on ice for 2hr. The cells were then washed twice in PBS and stained with PI solution (50µg/ml

propidium iodide (Invitrogen, Oregon, USA), 3.8mM sodium citrate, 0.5mg/ml RNase A) at room temperature for 30mins. DNA profile was analyzed using a flow cytometer (CyAn ADP Analyzer, Dako, Glostrup, Denmark). Cell cycle data analysis was performed by using the Summit^{TM} software (Dako, Glostrup, Denmark).

2.2.8 Cell migration assay

Quantitative cell migration was measured using Cytoselect^{TM} cell migration assay kit (Cell Biolabs, Inc, San Diego, CA, USA) according to manufacturer's protocol. Briefly, cells were serum starved for 24hr, harvested and seeded ($7.5x10^5$cells/ml) in triplicate in the upper chambers of the inserts. The inserts were then transferred into wells containing media with 10% of the chemoattractant, FBS and incubated for 20hr at 37°C. Migratory cells passed through polycarbonate membrane and clinged to the bottom side, while non-migratory cells stayed in the upper chamber. Upon removal of the later, migratory cells were stained and quantified at 570nm using Infinite F200 microplate reader.

2.2.9 Cell invasion assay

Cell invasion assay was assessed using QCM^{TM} 24-Well Cell Invasion Assay (Chemicon International, Temecula, CA, USA) with an 8 mm pore size according to the manufacturer's instructions. Briefly, cells were serum starved for 24h and harvested. Equal number of cells ($7.5x10^5$cells/ml) were seeded in triplicate onto the top chamber of the inserts and incubated at 37°C in a humidified 5% CO_2 atmosphere for 24h, with 10% FBS in the lower chamber. The cell suspension was then aspirated

while invading cells that had migrated through and attached to the bottom of membrane were then dissociated from membrane in cell detachment buffer. Cells were subsequently lysed and detected by CyQuant GR® dye. Fluorescence reading was taken at 485/535nm (Ex/Em) with Infinite F200 microplate reader.

2.2.10 Statistical analysis

All cell culture experiments were performed at least three times independently. Data for functional assays was presented as mean±SD as indicated. One-way analysis of variance or Student *t*-test was used to analyze the significance of differences. Two-tailed $p<0.05$ was considered significant.

Chapter 3: Results

3.1 Generation of ERp29-overexpressing MDA-MB231 and BT549 single stable clones

Gene transfection is one of the most utilized methods in unraveling protein functions. Thus, to elucidate the role of ERp29 in breast cancer progression, MDA-MB231 and BT549 breast cancer cells were stably-transfected with full-length of human ERp29 gene as described in Material and Methods. Upon isolation of single clones, the effectiveness of ERp29-transfection was verified by RT-PCR, immunoblot, as well as immunofluorescence assays (Figure 3-1 A, B, and C). Of the clones generated, two ERp29-overexpressing clones from each cell line were chosen for further experiments (Clone B and E for MDA-MB231, clone A and K for BT549). For simplification, the clones will be termed MDA-MB231/Ctrl, MDA-MB231/B, MDA-MB231/E, as well as BT549/Ctrl, BT549/A, BT549/K. Using β-actin as loading control, expression of ERp29 protein level revealed 3.2- and 4.6-fold of increase in MDA-MB231/B and MDA-MB231/E as well as 3.8- and 3.6-fold of increase in BT549/A and BT549/K compared to their control counterparts (Figure 3-1 B).

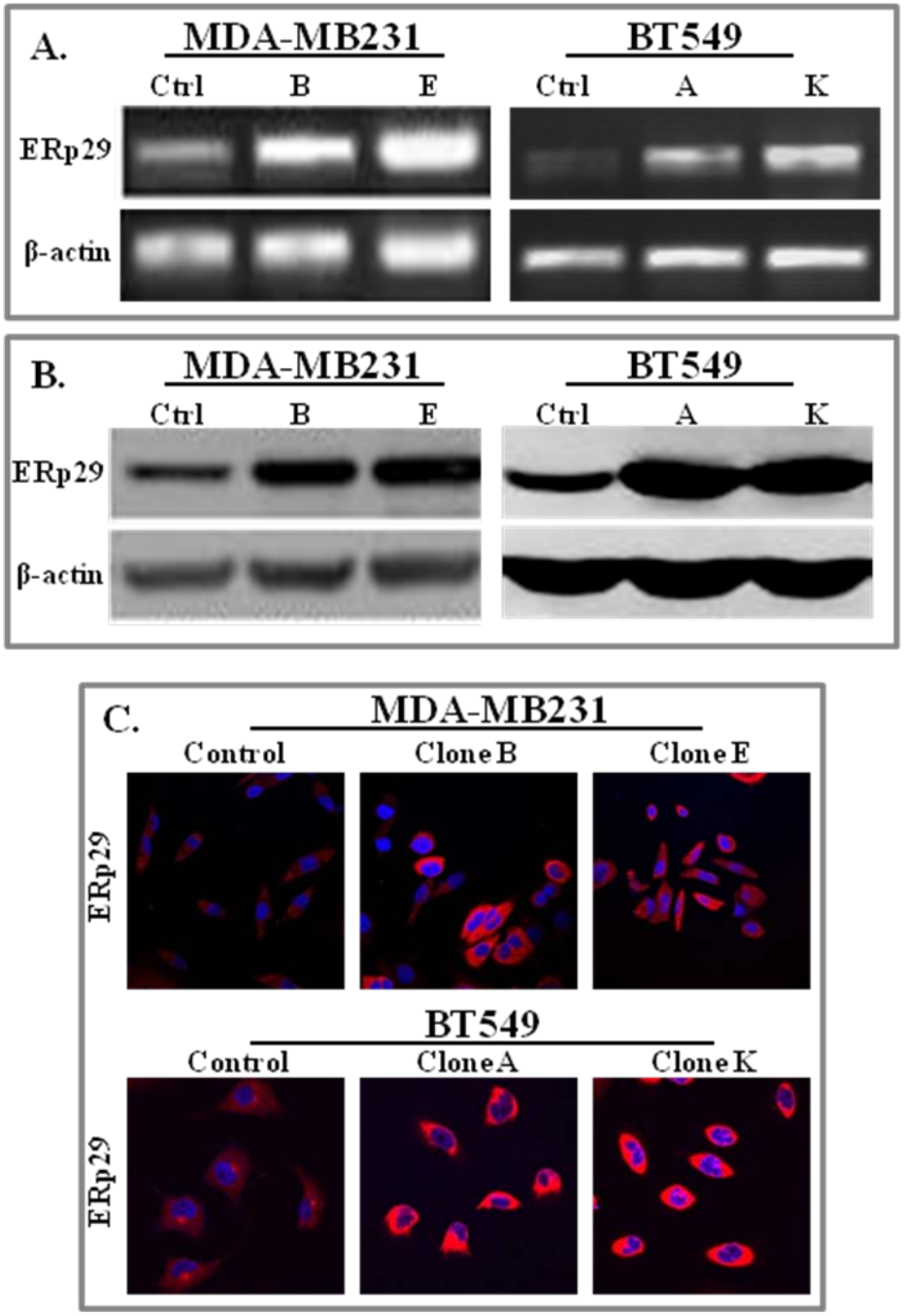


Figure 3-1 Expression of ERp29 in ERp29-transfected MDA-MB231 and BT549 cells. ERp29 expression in selected single stable clones was examined at mRNA (A) and protein level (B) by RT-PCR and immunoblot respectively. β-actin was used as loading control. Observations from immunofluorescence assay were in agreement with the immunoblot results (C). 4',6-diamidino- 2-phenylindole (DAPI-blue staining) was used to visualize DNA. (60× magnification)

3.2 Overexpression of ERp29 induces MET-morphological changes in MDA-MB231 and BT549 breast cancer cells

3.2.1 ERp29-overexpressing clones exhibit epithelial morphology

Upon isolation of single stable clones, significant morphological changes were observed in ERp29-overexpressing cells generated from both MDA-MB231 and BT549 cell lines. MDA-MB231/Ctrl exhibited mesenchymal-like morphology; elongated appearance, loose cell-cell contacts, and spreading morphology (Figure 3-2 A, top panel). In contrast, MDA-MB231/B and MDA-MB231/E, which often grew in clusters, displayed cobblestone appearance accompanied by increased cell-cell contacts. In addition, the actin cytoskeletal organization visualized by phalloidin staining demonstrated a change from filamentous stress fiber arrangement in control cells to cortical actin formation in ERp29 overexpressing MDA-MB231 clones (Figure 3-2 A, bottom panel). In agreement with the observed reduction of stress fiber, immunoblot and immunofluorescence assays performed showed decreased level of phosphorylated myosin light chain (pMLC) in MDA-MB231/B and MDA-MB231/E compared to the control (Figure 3-2 B and C). Phosphorylation of MLC by ROCK, a Rho-activated kinase downstream of RhoA activation, is necessary for the formation and maintenance of stress fiber (Sanders *et al.*, 1999).

Similarly, upon ERp29-transfection the morphology of BT549 cells was altered. BT549/Ctrl had elongated spindle-like appearance characteristic of basal-like cells, while BT549/A and BT549/K showed a more epithelial morphology indicated by compact organization (Figure 3-2 A, top panel). It should be noted that the extent of

ERp29-induced morphological change was more prominent in MDA-MB231 than what was observed in BT549 cells. Further inspection on the cytoskeletal organization also demonstrated a change from filamentous stress fiber to cortical actin formation (Figure 3-2 A, bottom panel). In accordance, level of pMLC was reduced in ERp29-overexpressing BT549 clones compared to their control counterpart (Figure 3-2 B and C). Akin to the degree of morphological changes, the pMLC reduction was more apparent in ERp29-overexpressing MDA-MB231 clones than in ERp29-overexpressing BT549 clones.

Taken together these results suggest that overexpression of ERp29 induces reversion of mesenchymal MDA-MB231 and basal-like BT549 breast cancer cells to a phenotype that resembles epithelial cells, which is reminiscent of morphological aspect of MET.

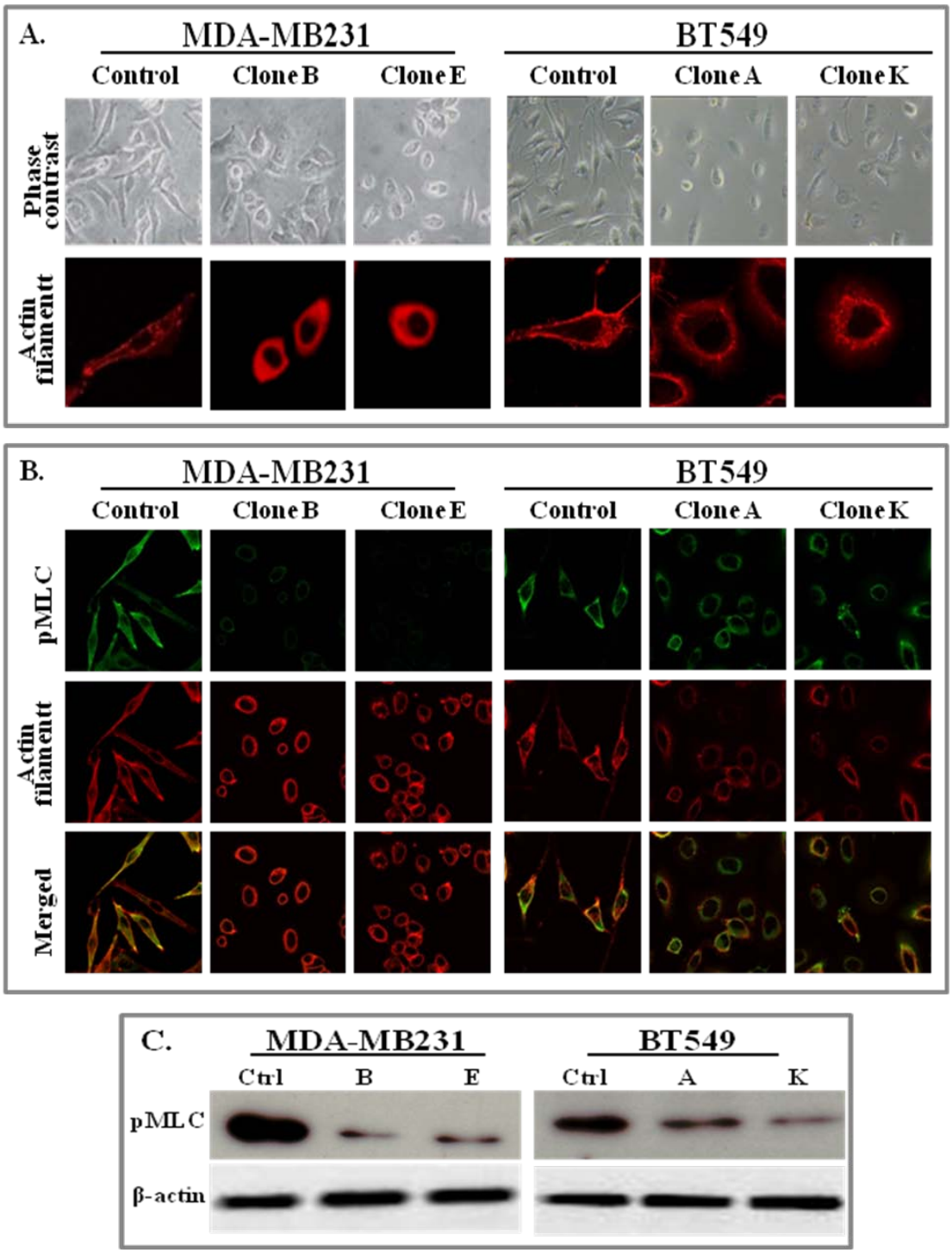


Figure 3-2 Morphological changes and cytoskeletal actin rearrangement in ERp29-overexpressing MDA-MB231 and BT549 clones. (A) Top panel: comparison of morphological changes between control cells and ERp29-overexpressing clones under phase contrast microscope (60× magnification). Bottom panel: cytoskeletal actin remodeling from stress fiber to submembranous cortical actin as visualized by rhodamine phalloidin staining (120X magnification). (B) Immunofluorescence examined the decrease in phosphorylation of MLC (green), consistent with the observed reduction of stress fiber formation (60X magnification). (C) Verification of pMLC level by immunoblot was in agreement with immunofluorescence assay. β-actin was used as loading control.

3.2.2 Overexpression of ERp29 restrores tight junctions and cell polarization

Epithelial morphology is maintained by proteins involved in cell-cell junctions and cell polarization. Therefore, expression of members of tight junctions and polarity complexes were examined by RT-PCR and immunoblot. In addition, immunofluoresence assay was performed to analyze their cellular distribution and expression.

Both apical polarity complexes, Par and Crumbs, are important in recognizing initial polarization cues and establishment of junctional complexes. However, Par complex-associated proteins (Par3, Par6, aPKC) were chosen to be investigated as compared to Crumbs, since Par complex is involved in broader range of cell types and regulates more diverse polarity-related cellular events (Wang *et al.*, 2007). Additionally, Scribble (care-taker of the basolateral identity in cell polarization) as well as ZO1 (marker of tight junctions development) were also examined.

Initial work by RT-PCR revealed that all of the genes tested remain significantly unchanged at transcriptional level both in MDA-MB231 and BT549 models (Figure 3-3 A). Figure 3-3 B however, shows that compared to the control cells, there was an increase of protein level of Par3 and Scribble in ERp29-overexpressing MDA-MB231 and BT549 clones, indicating post-transcriptional regulation of these proteins. The level of Par6 and aPKC remained significantly unaltered.

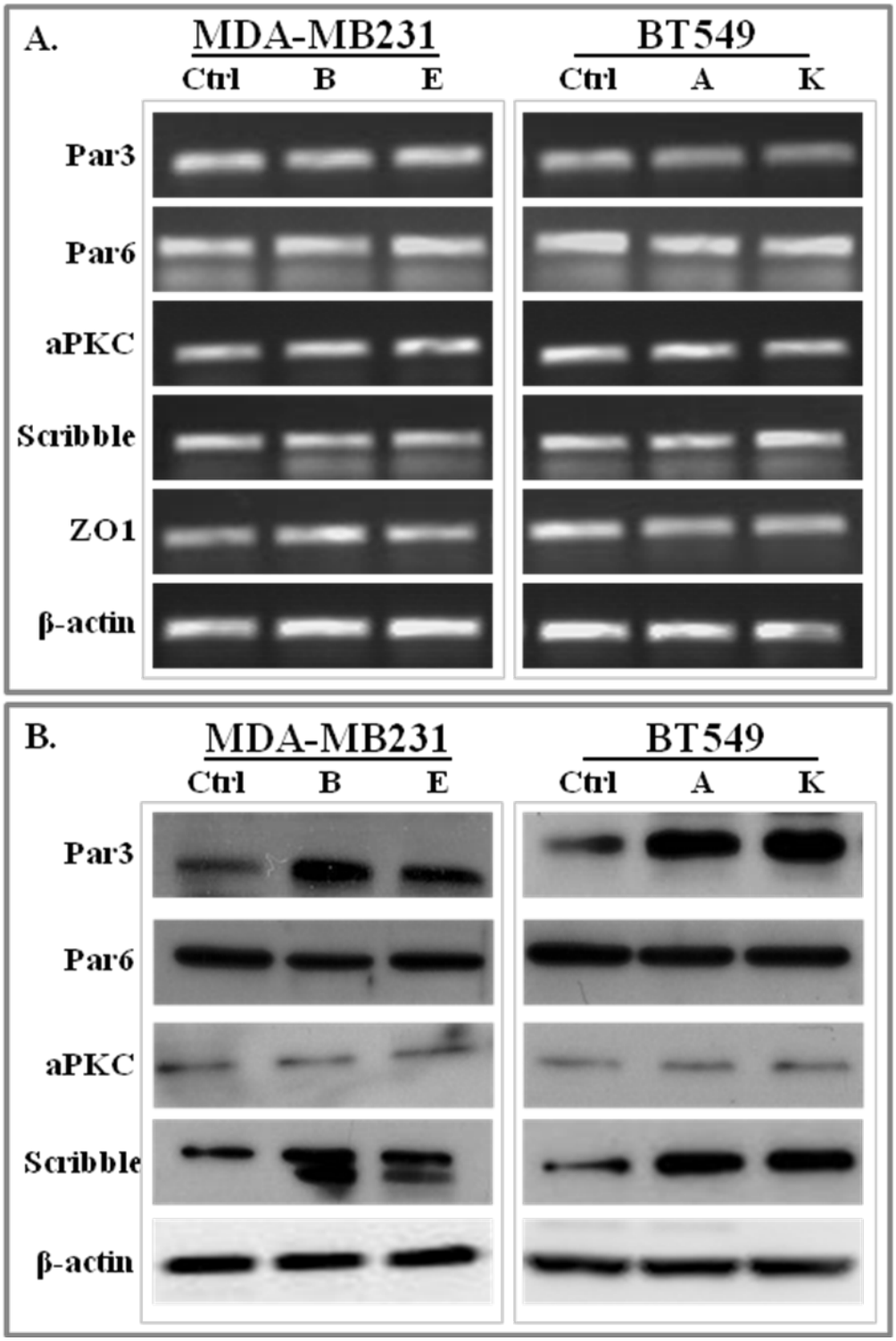


Figure 3-3 Overexpression of ERp29 regulated tight junction and polarity proteins at protein level. (A) mRNA level of all the investigated proteins remained unchanged. (B) Protein expression of Par3 and Scribble was increased in ERp29-overexpressing MDA-MB231 and BT549, but that of Par6, and aPKC was unaltered. β-actin was used a loading control.

Par3 is known as one of the early signal in the establishment of apical-basal polarity (reviewed in Godde *et al.*, 2010). Initiation cues induce its proper relocalization where it then promotes separation of apical and basal polarity complexes as well as enrichment of cell-cell junction modulators. Figure 3-4 A shows that while Par3 exhibited diffused expression in MDA-MB231/Ctrl, it was localized at cell-cell contact sites in MDA-MB231/B and MDA-MB231/E, indicating its functional state. In contrast, there was no change in localization of Par6 and aPKC. Basal polarity protein Scribble was also properly localized in ERp29-overexpressing MDA-MB231 clones (Figure 3-4 A). As mentioned earlier, formation of tight junctions is often considered as the final step of MET (Lee *et al.*, 2006). Thus membranous staining of ZO1 suggests that MDA-MB231/B and MDA-MB231/E has undergone full MET and completed cell polarity program. This proposal, however, must be considered with care as in carcinogenesis sequential order of developmental EMT/MET is often not followed closely.

In ERp29-overexpressing BT549 clones, similar membrane redistribution of Scribble, as in ERp29-overexpressing MDA-MB231 clones, was observed (Figure 3-4 B). However, ZO1 and Par3 seemed to be affected in lesser extent. Instead of continuous staining, ZO1 in BT549/A and BT549/K showed spot-like nascent tight junctions. Similarly, the expression of Par3 in ERp29-overexpressing BT549 clones was detected only in some of the cell-cell contact sites (indicated by white arrows). Similar to MDA-MB231 model, distribution of other Par complex, Par6 and aPKC, remained unchanged in ERp29-overexpressing BT549 clones compared to the control

cells.

While Par3 is known to function in the establishment of cell polarization, Scribble is believed to be involved in the maintenance of apical-basal polarity as well as cell junctions (Qin *et al.,* 2005). Its downregulation in Madin-Darby Canine Kidney (MDCK) cells results in delay in ZO1 assembly (Qin *et al.,* 2005). In both ERp29-overexpressing MDA-MB231 and BT549 clones, Scribble is properly localized at cell membrane (Figure 3-4). While this observation does not follow the sequential order of apical-basal polarity establishment, it supports that to certain degree that ERp29 does restore cell polarization. Furthermore, Scribble is a known tumor suppressor, which is targeted by E6 protein of human papillomavirus, a major cause of cervical cancer (Nakagawa *et al.,* 2000). In breast cancer, Scribble is often downregulated or mislocalized, and it has been shown to inhibit breast cancer progression in MCF10A cells (Zhan *et al.,* 2008). Therefore, it can be suggested that its upregulation contributes to *in vivo* tumor suppressing effect of ERp29 (Bambang *et al.,* 2009b).

Taken together, the observations of the phenotypic changes and the relocalization of junctional Par3, Scribble, and ZO1 in ERp29-overexpressing MDA-MB231 and BT549 clones indicate that these cells have undergone reversion to a phenotype that displays aspects of epithelial polarity, further supporting the notion of ERp29-induced morphologic MET.

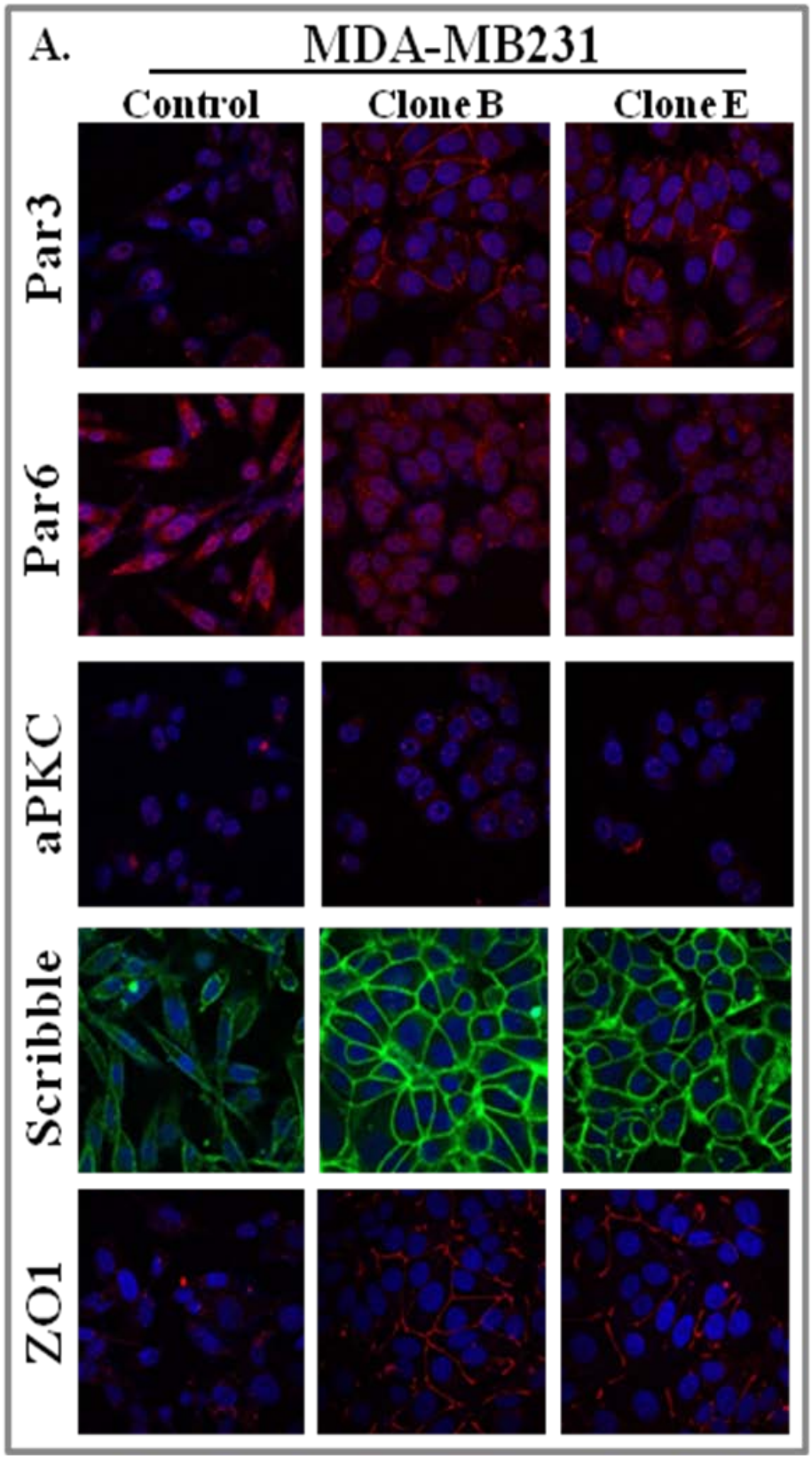
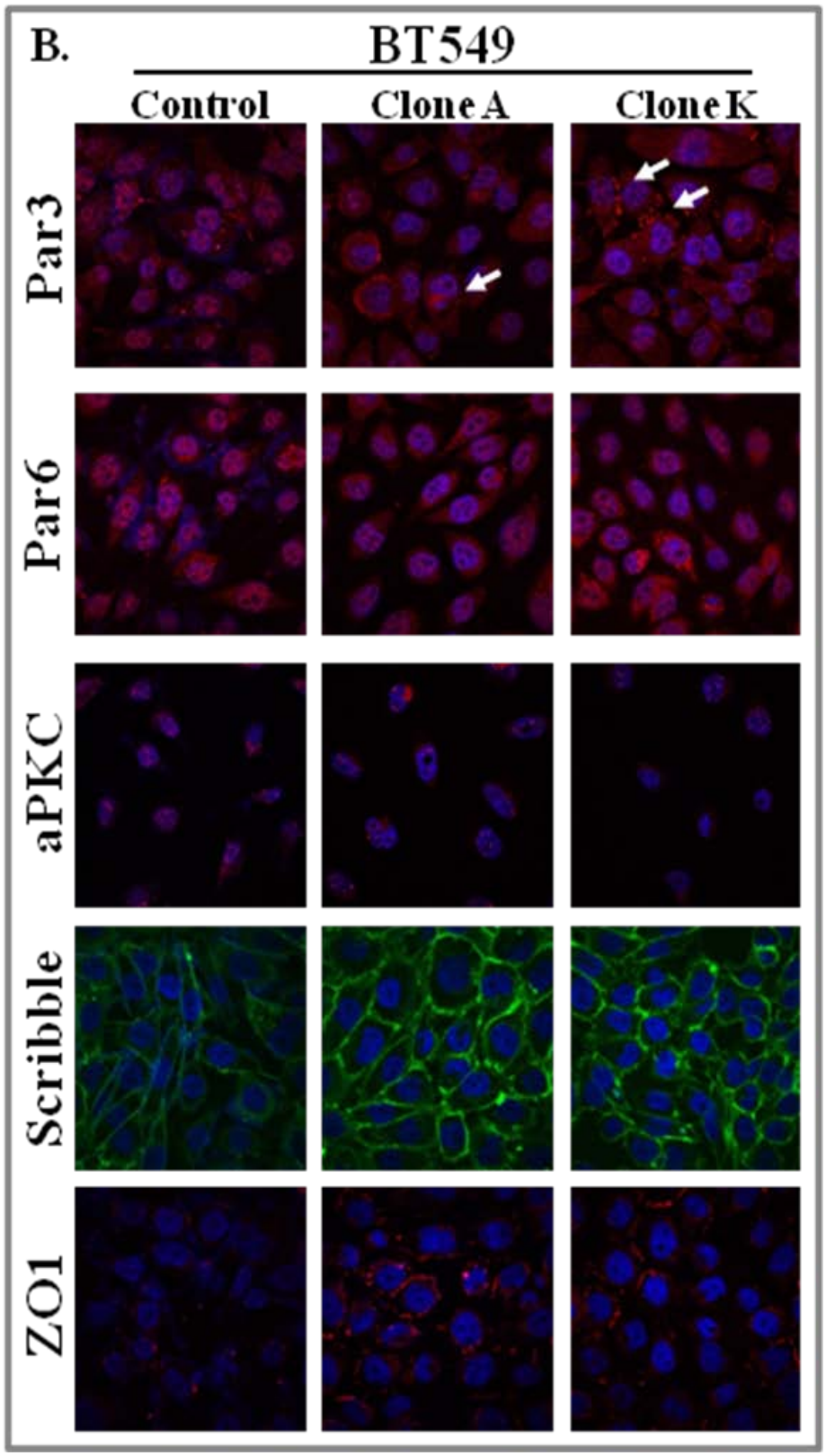


Figure 3-4 Overexpression of ERp29 relocalized Par3, Scribble, and ZO1 to cell-cell contact sites. (A) Immunoflourescence assay showed strong localization of Par3, Scribble, and ZO1 at the cell-cell contact sites in MDA-MB231/B and MDA-MB231/E, while control cells displayed diffused pattern. DAPI was used to stain nucleus (blue) (60× magnification).



Cont'd **Figure 3-4 Overexpression of ERp29 relocalized Par3, Scribble, and ZO1 to cell-cell contact sites.** (B) Scribble showed a strong membranous staining in ERp29-overexpressing BT549 clones. Par3 and ZO1 were also detected in the cell periphery though with weaker signal (white arrows). DAPI was used to stain nucleus (blue) (60× magnification).

3.2.3 Overexpression of ERp29 inhibits cell proliferation

Changes in cell shape are often associated with physiological alterations, including proliferative potential (Lu *et al.*, 2001). To examine whether overexpression of ERp29 regulates proliferation of MDA-MB231 and/or BT549 cells, cell growth assay over a four-day period was performed. Figure 3-5 A shows negative regulation of cell proliferation in ERp29-overexpressing MDA-MB231 clones compared to the control cells. This ERp29-induced inhibition of proliferation in MDA-MB231 is implicated in tumor growth suppression *in vivo* (Bambang *et al.*, 2009b). Likewise, BT549/A and BT549/K grew at slower rate compared to BT549/Ctrl (Figure 3-5 B).

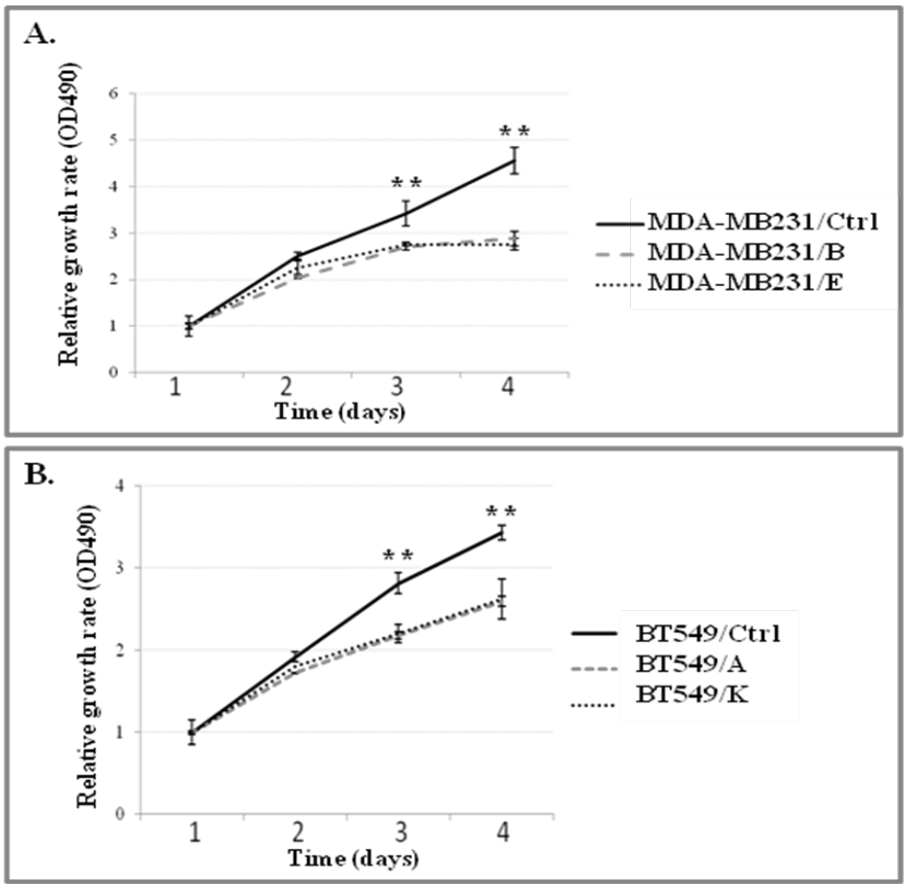


Figure 3-5 Overexpression of ERp29 inhibited cell proliferation. Both ERp29-overexpressing MDA-MB231 (A) and BT549 clones (B) demonstrated slower proliferation rate compared to the control cells. Data shown represents the mean±SD of triplicate experiment. Bars, SD; **p<0.05.

The effect of cell morphology on proliferative potential is contributed by its influence on cell cycle control. Depending on the shape, cell may or may not be able to acquire signals from its environment to pass through cell cycle restriction point (Huang *et al*., 1998). Cell spreading allows signals elicited by growth factors from the environment to pass through G1/S restriction point while compact morphology prevents growth factor access thereby inducing cell cycle arrest at G1/S.

To learn if overexpression of ERp29 inhibits cell proliferation by regulating cell cycle progression, cell cycle profile was obtained for control and transfected-cells using propidium idodide which visualized DNA contents. Figure 3-6 A shows increase of G0/G1 population in ERp29-transfected MDA-MB231 compared to the control, indicating ERp29-induced cell cycle arrest at the stage. In accordance with this finding, further inspections revealed that cyclin-dependent kinase inhibitor 2B (CCKN2B, p15), a G1 checkpoint protein, was activated in MDA-MB231/B and MDA-MB231/E (Bambang *et al.*, 2009b).

Surprisingly, similar observations were not observed in the BT549 model. BT549/A and BT549/K had increased G2/M population compared to BT549/Ctrl implying G2/M arrest (Figure 3-4 B). This indicated that ERp29-transfected BT549 employed a different mechanism in regulating cell proliferation which warrants further investigations. In some cases, the ability to regulate morphology can be uncoupled form ability to modulate cell proliferation (Aranda *et al.*, 2006). Therefore, this has to be taken under consideration in examining the nature of proliferation inhibition by ERp29.

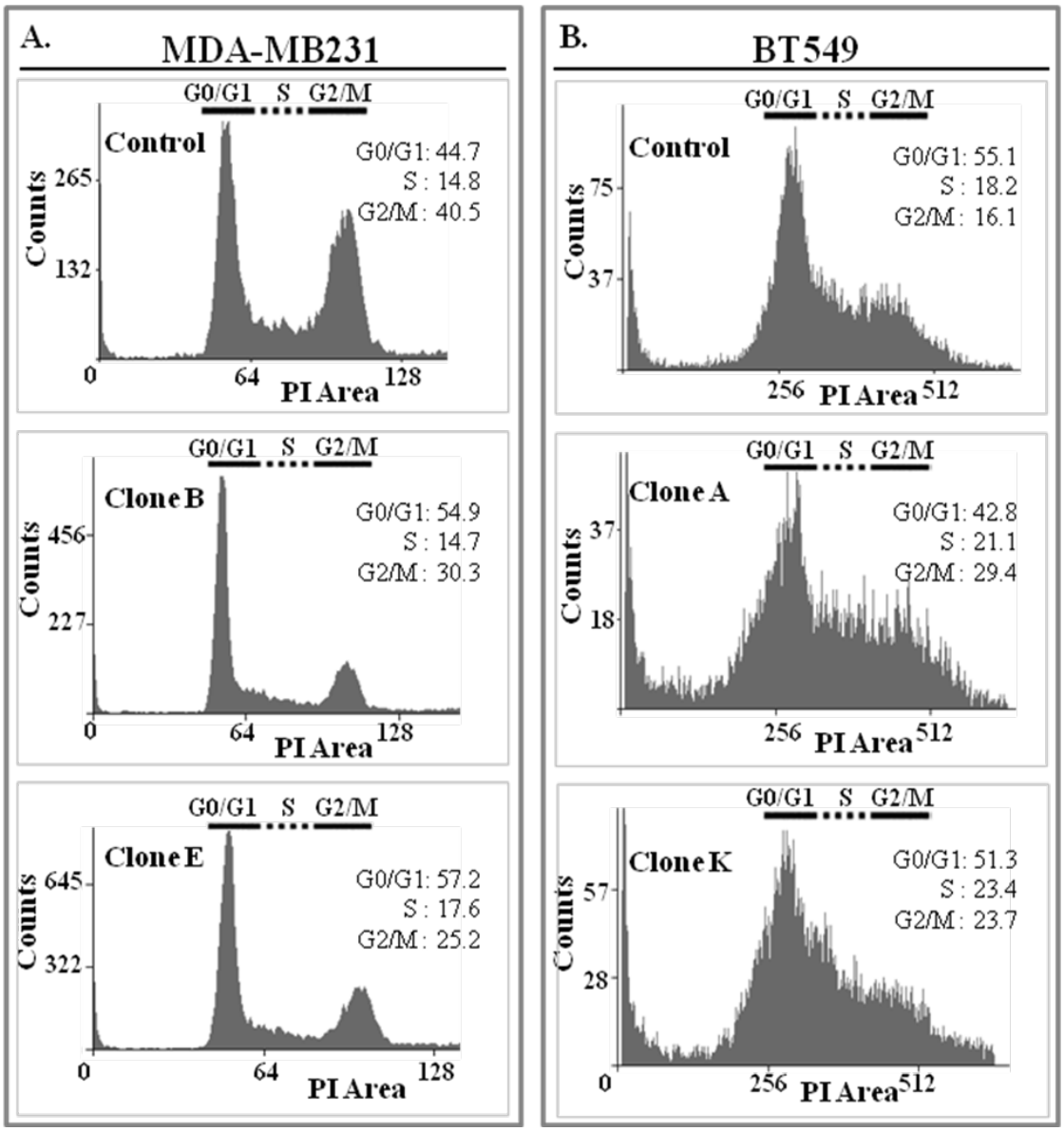


Figure 3-6 Overexpression of ERp29 regulated cell cycle progression. Representative cell cycle profile of MDA-MB231/B and MDA-MB231/E (A), as well as BT549/A and BT549/K (B) with their respective controls. Note the increase of G0/G1 populations in ERp29-tranfected MDA-MB231 and G2/M populations in ERp29-transfected BT549.

Thus far, the results suggest that overexpression of ERp29 in MDA-MB231 and BT549 breast cancer cells induces alterations in cell shape, reminiscent of morphological changes in MET, accompanied by inhibition of cell proliferation whose mechanisms might involve regulation of cell cycle progression.

3.3 Overexpression of ERp29 induces MET-molecular changes in MDA-MB231 cells

3.3.1 Regulation of EMT/MET markers.

The finding that ERp29-overexpression in MDA-MB231 and BT549 cells resulted in morphological change led to the question whether or not the change is associated with molecular MET. Thus, the expression and localization of epithelial markers and mesenchymal markers were examined using immunoblot and immunofluorescence analysis. Expression of E-cadherin and cytokeratin-19 (CK19), the primary intermediate filament in mammary epithelial cells, was used as markers for epithelial phenotype; as well as expression and more importantly membranous localization of β-catenin. Expression of vimentin and fibronectin was employed as mesenchymal markers.

As indicated in Figure 3-7 A (left panel), MDA-MB231/B and MDA/MB231/E showed an upregulated levels of epithelial markers (E-cadherin, CK19, and total β-catenin) and downregulated levels of mesenchymal markers (fibronectin and vimentin) compared to MDA-MB231/Ctrl. Further investigation revealed that the upregulated E-cadherin is functional as it was relocalized at cell membrane (Figure 3-7 B, left panel). Overexpression of ERp29 also regulated intracellular distribution of β-catenin which is a determinant of cell fate (Figure 3-7 B, left panel). As mentioned earlier, β-catenin is a dual-function protein; membranous β-catenin mediates contact of E-cadherin and actin cytoskeleton while nuclear β-catenin binds to TCF/LEF and acts as transcription factor. This complex mediates Wnt signaling and is responsible

for cell proliferation, dedifferentiation, inhibition of apoptosis, and tumor progression by inducing EMT (Reva *et al.*, 2005; van de Wetering *et al.*, 2002; Fuchs *et al.*, 2005). In contrast, reduction of β-catenin/TCF/LEF activity has been demonstrated to promote epithelial differentiation (van de Wetering *et al.*, 2002). Thus, in addition to supporting the notion of molecular MET in ERp29-overexpressing MDA-MB231 clones, relocalization of β-catenin from nucleus to cell membrane suggests that ERp29 signaling crosstalk to the Wnt signaling.

Interestingly, similar observations were not found in the BT549 model (Figure 3-7 A and B, right panel). Overexpression of ERp29 did not regulate the expression and/or localization of E-cadherin, CK19, and vimentin, while expression of β-catenin was slightly reduced in BT549/A and BT549/K compared to BT549/Ctrl. It is worth noting that while overexpression of ERp29 did not change intracellular distribution of β-catenin in this cell line, localization of this protein was different in MDA-MB231/Ctrl and BT549/Ctrl (Figure 3-7 B). β-catenin is mainly localized in the nucleus in MDA-MB231 cells, whereas β-catenin staining is diffused in cytoplasm with slightly stronger membranous signal in BT549 cells. These suggest that β-catenin functions differently in the different cell lines; it does not mediate Wnt signaling in BT549 cells. It is also interesting to note that the mesenchymal marker fibronectin was significantly downregulated in ERp29-overexpressing BT549 clones compared to their control counterparts. Fibronectin is a component of extracellular matrix that is generated by mesenchymes and not epithelia. Its production enhances growth factor-induced EMT in primary human bronchial epithelial cells (Câmara *et al.*, 2010).

Furthermore, the upregulation of fibronectin has been shown to be one of the early events in EMT without apparent reduction of E-cadherin, suggesting the existence of a separate mechanism regulating its expression (Lindley *et al.*, 2010). The persistence of mesenchymal markers and failure to fully express epithelial markers in ERp29-overexpressing BT549 compared to MDA-MB231 clones suggests that BT549 cells transfected with ERp29 still maintained some aspects of mesenchymal phenotype. Further investigations of other EMT markers, such as other cytokeratins, α-smooth muscle actin, are needed to determine the extent of the transition in ERp29-overexpressing BT549 cells.

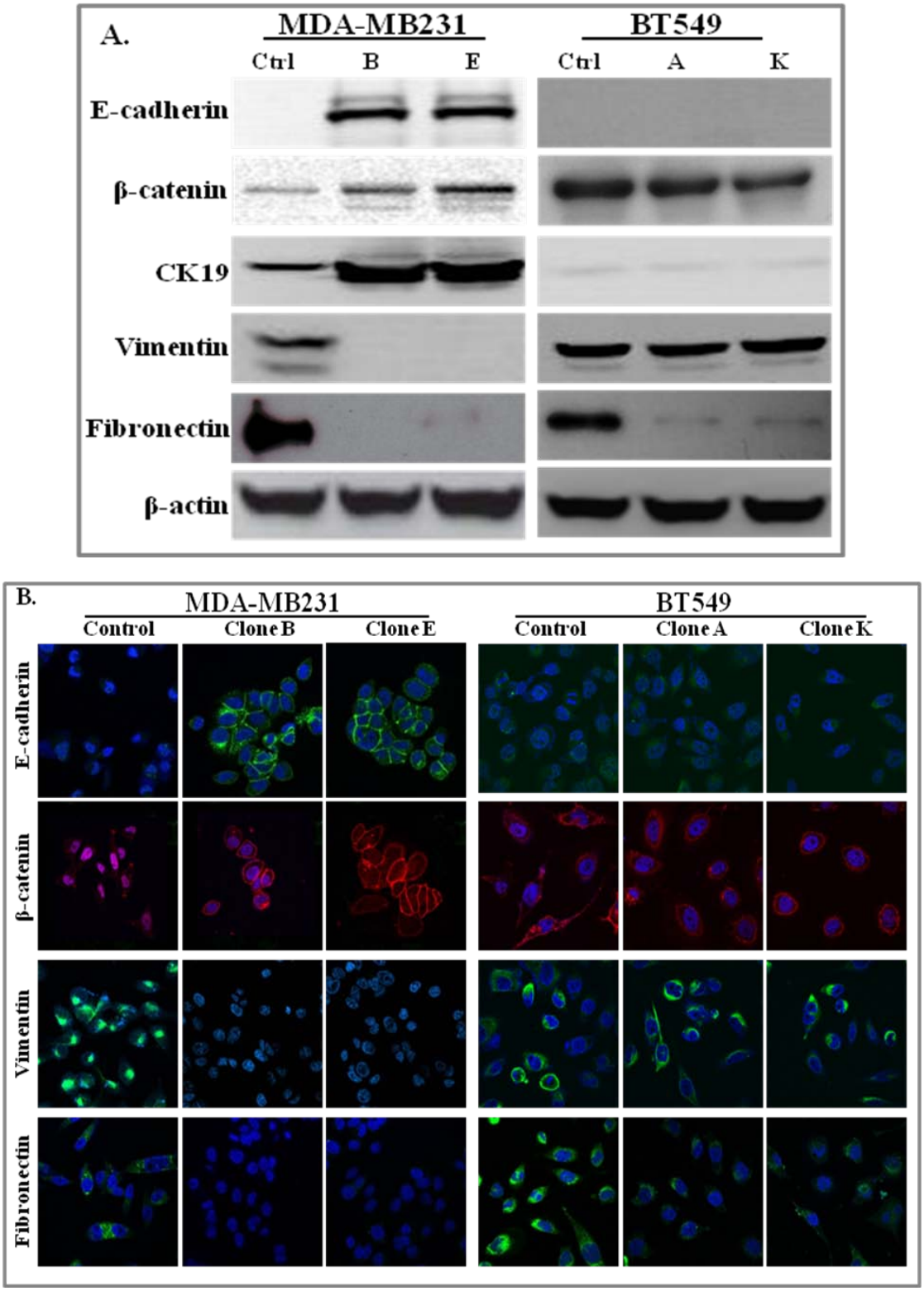


Figure 3-7 Profile of epithelial and mesenchymal markers in ERp29-overexpressing MDA-MB231 and BT549 clones. (A) Protein level of epithelial markers (E-cadherin, β-catenin, and CK19) and mesenchymal markers (vimentin and fibronectin) as examined by immunoblot. β-actin was used as loading control. (B) Immunofluorescence images revealed the localization of EMT/MET markers. DAPI staining (blue) indicated the nuclear staining. (60× magnification)

3.3.2 Regulation of E-cadherin repressors.

Molecular alterations of MET are largely contributed by transcription factors that regulates EMT/MET markers, in particular E-cadherin. To investigate this, the kinetics of known E-cadherin regulators (SNAI1, SNAI2, ZEB1, ZEB2, TCF3, and Twist) were investigated by RT-PCR and immunoblot. In addition, immunofluorescence assay was performed to learn the localization of the regulators as their transcriptional activity also depends on subcelullar localization. For example, phosphorylation of SNAI1 by p21-activated kinase-1 (PAK1) results in nuclear accumulation of SNAI1 and subsequent transcriptional repression of target genes (Thiery *et al.*, 2006). On the other hand, knockdown of PAK1 expression resulted in cytoplasmic accumulation of SNAI1 and ablation of its transcriptional-repressor activity.

Concurrent with the upregulation of E-cadherin observed earlier, the transcription repressors SNAI1, SNAI2, ZEB2, and Twist were downregulated both at transcriptional and translational levels in ERp29-overexpressing MDA-MB231 clones compared to the control cells (Figure 3-8 A). RT-PCR and immunoblot revealed unchanged expression level of ZEB1, excluding its involvement in ERp29-mediated downregulation of E-cadherin in MDA-MB231 cells. Of note, there was no change in the localization of all of the transcription factors (Figure 3-9 left panel).

Also in agreement with previous results, overexpression of ERp29 did not negatively regulate the level of any E-cadherin repressors in BT549 cells (Figure 3-8 B) nor did it change their localization (Figure 3-9, right panel). These suggested that SNAI1,

ZEB1, ZEB2, and Twist were not affected by ERp29-overexpression in BT549 cells. Furthermore there was an increase of SNAI2 protein but not its mRNA in BT549/A and BT549/K compared to BT549/Ctrl. Contrast to SNAI1, SNAI2 is more broadly expressed and in some cases its expression is unrelated to EMT (Barrallo-Gimeno *et al.*, 2005). Due to its subnuclear localization, SNAI2 has been postulated to localize to target promoters, where activation occurs, to repress basal and activator-mediated transcription (Hemavathy *et al.*, 2000).

It is interesting to note that TCF3 was upregulated in both ERp29-overexpressing MDA-MB231 and BT549 clones. The upregulation was observed at both transcriptional and translational levels (Figure 3-8). Although the biochemical reason is unclear, this phenomenon is not unprecedented as there exist examples where E-cadherin repressors do not show inverse relation with E-cadherin levels (Rosivatz *et.al.*, 2002; Conacci-Sorrell *et.al.*, 2003). TCF3 has been shown to be downregulated in immortalized breast cells MCF10A undergoing spontaneous EMT, while SNAI2 was upregulated (Sarrió *et al.*, 2008).

Taken together, the results suggest that downregulation of E-cadherin repressors is responsible, at least in part, for the molecular alterations of ERp29-induced molecular MET in MDA-MB231 cells.

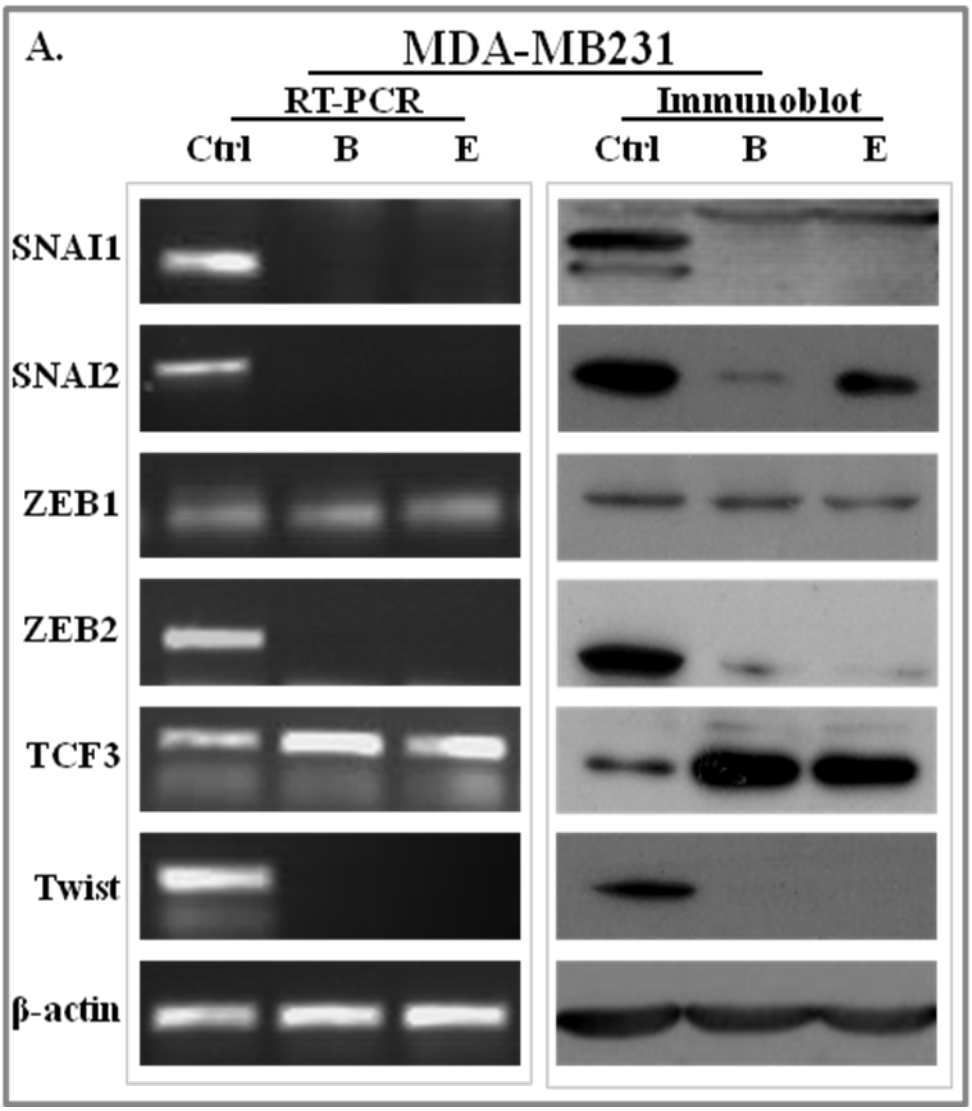
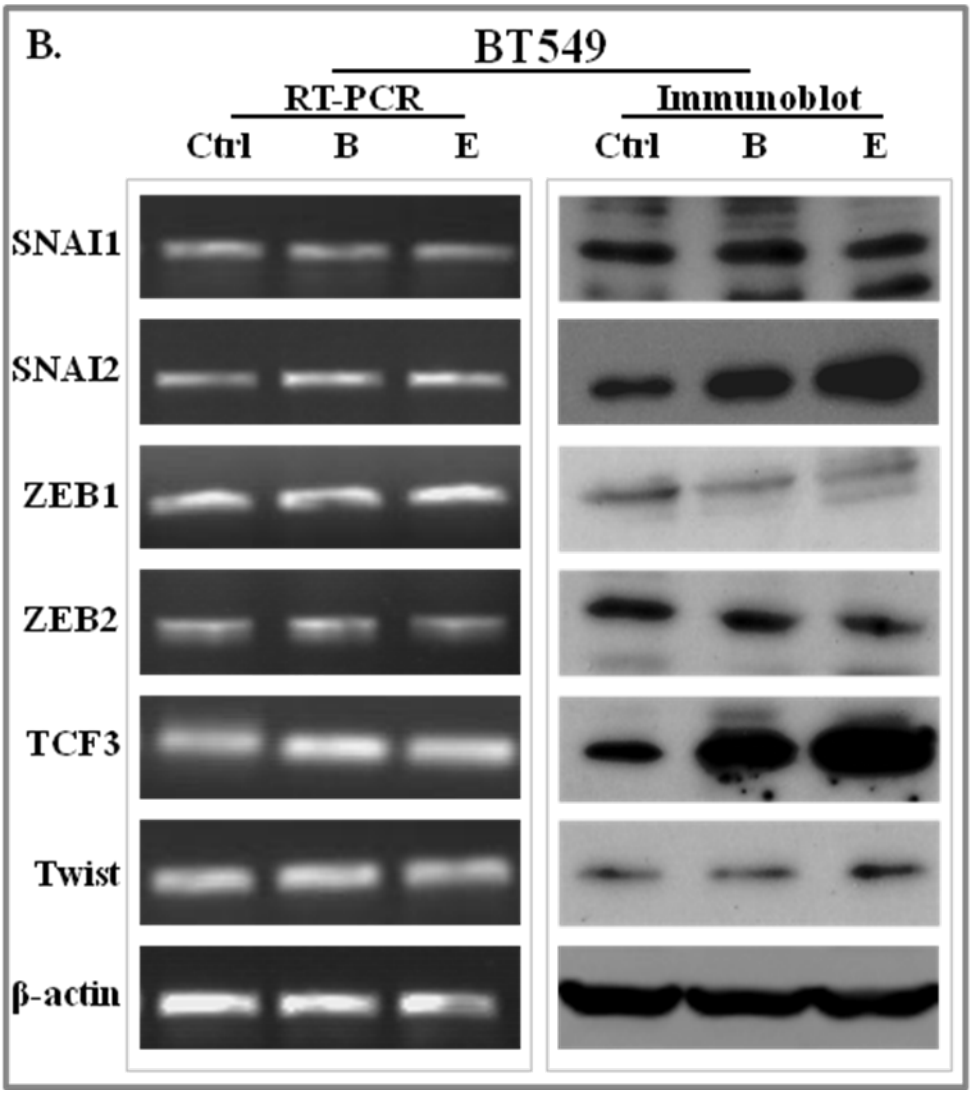


Figure 3-8 Overexpression of ERp29 differently regulated E-cadherin repressors in MDA-MB231 and BT549 cells. (A) ERp29-overexpressing MDA-MB231 clones expressed lower level of SNAI1, SNAI2, ZEB2, and Twist at transcriptional (left panel) and translational level (right panel) compared to control cells; while expression of TCF3 was increased. β-actin was used as loading control.



Cont'd **Figure 3-8 Overexpression of ERp29 differently regulated E-cadherin repressors in MDA-MB231 and BT549 cells.** (B) Overexpression of ERp29 did not downregulate any of the E-cadherin repressors in BT549 cells. Note that in both ERp29-overexpressing MDA-MB231 and BT549 clones, transcriptional and translational level of TCF3 was upregulated. β-actin was used as loading control

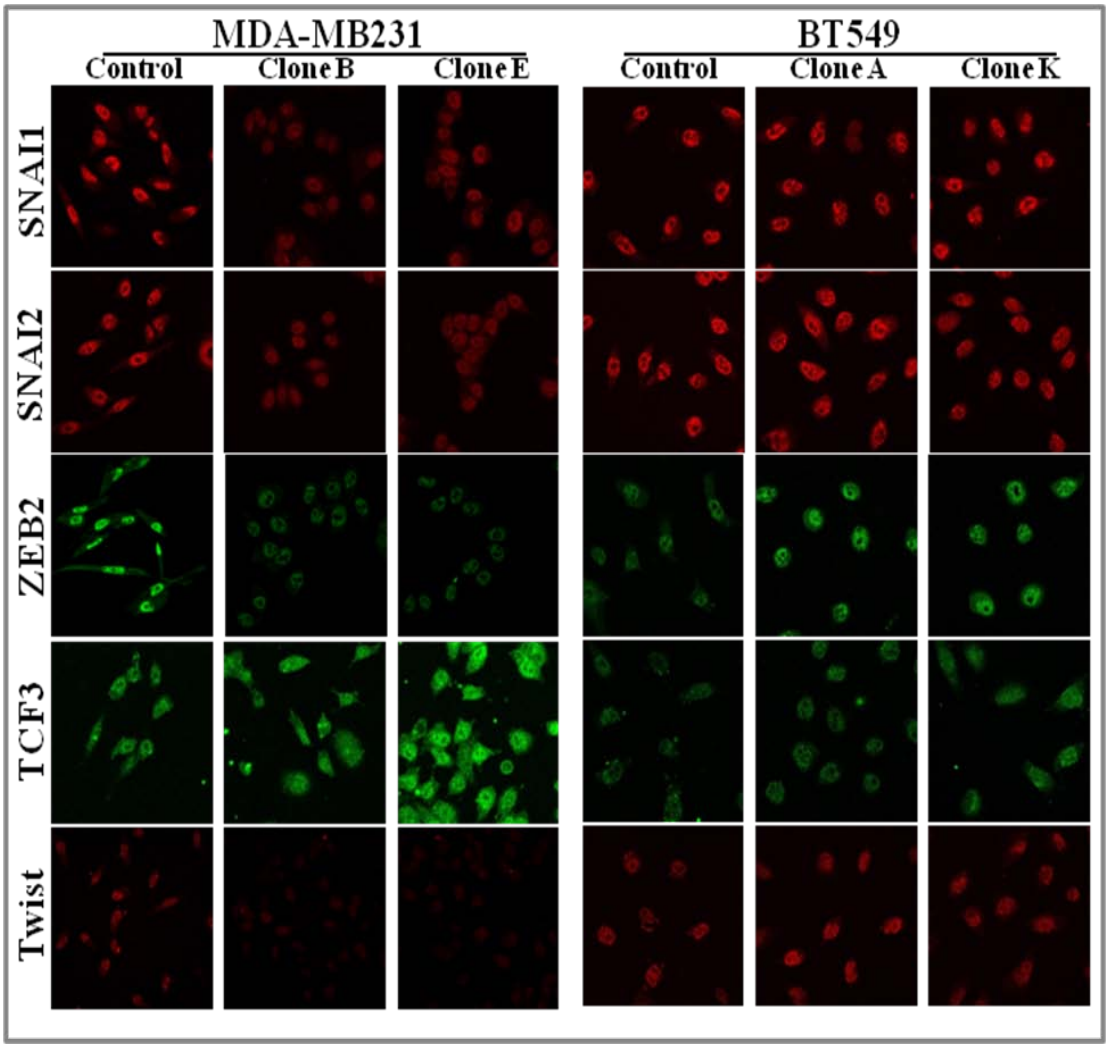


Figure 3-9 Overexpression of ERp29 did not alter the localization of E-cadherin repressors. Immunofluorescence analysis confirmed the dowregulation of SNAI1, SNAI2, ZEB2, and Twist in MDA-MB231/B and MDA-MB231/E compared to MDA-MB231/Ctrl. In addition, there was no relocalization of any of the E-cadherin repressors tested both in MDA-MB231 (left panel) and BT549 cells (right panel). (60× magnification).

3.4 Overexpression of ERp29 induces MET-behavioral changes in MDA-MB231 cells

In addition to morphological and molecular alterations, MET also confers behavioral changes characterized by decrease in cell motility and invasiveness. Therefore, migration and invasion ability was examined in ERp29-overexpressing MDA-MB231 and BT549 cells.

Quantitative analysis using Transwell chamber showed a reduced motility in MDA-MB231/B and MDA-MB231/E compared to their control counterpart. Similar trend was also observed in the Matrigel invasion assay. As shown in Figure 3-10 A and B, both invasiveness and migration were reduced to less than 50% upon overexpression of ERp29. The cytoplasmic distribution of β-catenin and downregulation of SNAI1 might be accountable for this observation as nuclear localization of the first and expression of the second are known to regulate MMPs.

Figure 3-10 C and D indicate that no significant change of neither migration nor invasion capacity was evident in ERp29-overexpressing BT549 clones compared to control cells. These observations were not unprecedented as behavioral change is closely associated with transcriptional reprogramming of EMT/MET which was not observed in this model (Figure 3-7 and 3-8 B). Downregulation of E-cadherin, in particular, is known to be essential for regulation of motility and invasiveness during EMT/MET (Maeda *et al.*, 2005; Lindley *et al.*, 2010).

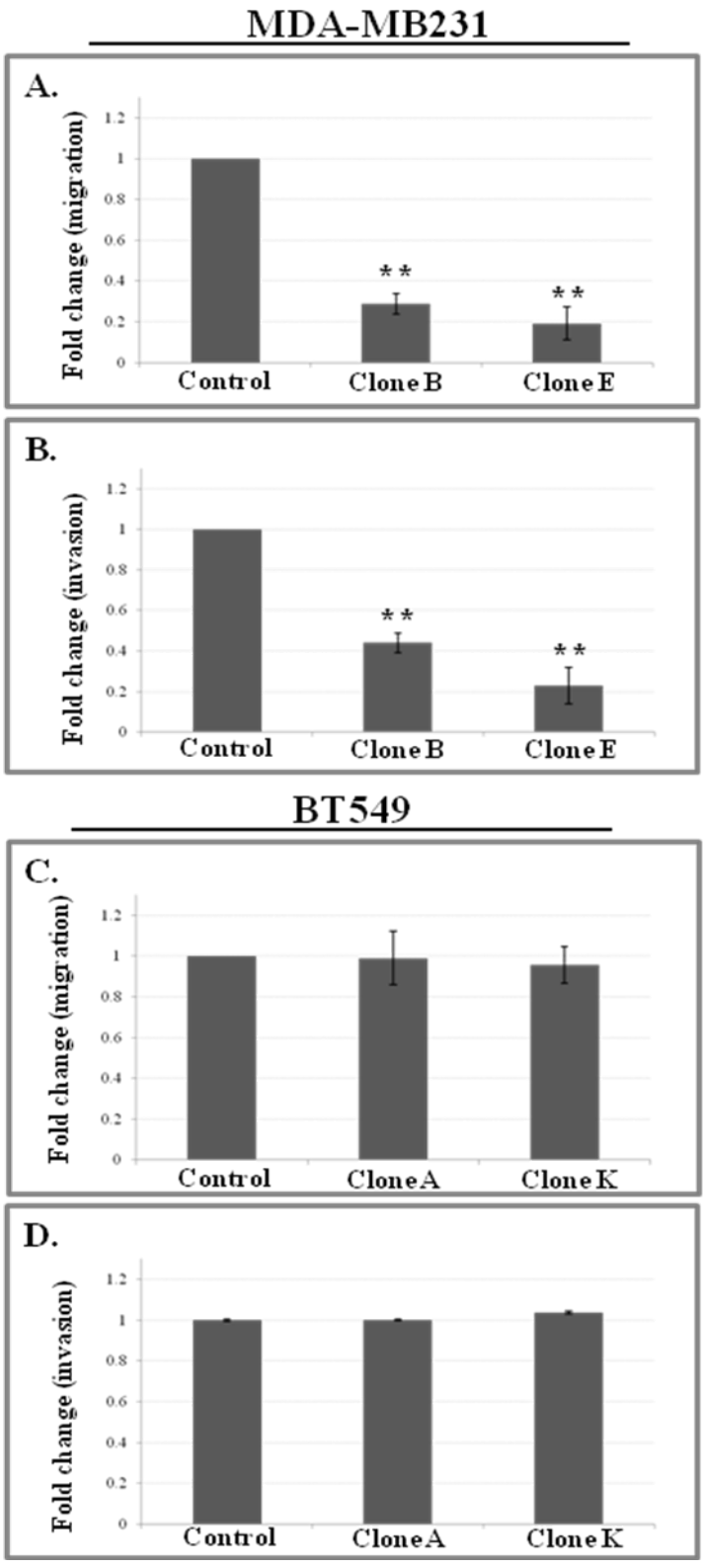


Figure 3-10 Overexpression of ERp29 reduced motility and invasiveness of MDA-MB231 cells but not BT549 cells. Migration and invasion potential in ERp29-overexpressing MDA-MB231 (A, B) and BT549 clones (C, D) compared to control cells. Results were expressed as a mean of fold change from three independent results. Bars, SD; **$p<0.05$.

In summary, results presented here indicate that overexpression of ERp29 induced MET in both MDA-MB231 and BT549 cells. There are, however, discrepancies in the degree of ERp29-induced MET in the two models. In MDA-MB231 cells, overexpression of ERp29 results in complete MET characterised by all three aspects; morphological, molecular and behavioral. In contrast, overexpression of ERp29 in BT549 cells only confers morphological MET. In the next chapter, the novel finding of ERp29-induced MET in breast cancer cells will be discussed in greater details.

Chapter 4: Discussions

Recently it has been reported that ERp29 expression is downregulated in breast tumors and is conversely related with breast cancer progression, suggesting a role of ERp29 as tumor suppressor (Bambang *et al.*, 2009b). To address the functional contributions of ERp29 in breast cancer progression, MDA-MB231 and BT549 invasive breast cancer cells, are transfected with full-length ERp29. In mesenchymal-like MDA-MB231 cells, overexpression of ERp29 results in complete MET characterized by changes in morphological (from mesenchymal to epithelial-like appearance), molecular (transcriptional regulation of EMT/MET markers), and behavioral changes (reduced capacity of migration and invasion). In basal-like BT549 cells, ERp29 overexpression induces morphologic MET, which is similar to what happens in MDA-MB231 cells, is associated with cytoskeletal rearrangement and restoration of tight junctions and cell polarization. The change observed in ERp29-overexpressing BT549 cells is not accompanied by transcriptional and translational changes of E-cadherin, CK19, and vimentin, although fibronectin, one of the mesenchymal markers, is decreased. These data indicate the overexpression of ERp29 in BT549 cells led to incomplete MET.

In this chapter, discussions will be focused on: (1) possible explanations for the discrepancies found in the two cell lines, (2) plausible mechanisms that lead to ERp29-induced MET, and (3) the contribution of this study in unraveling the functions of ERp29 especially in breast cancer progression.

4.1 Breast cancer cells: MDA-MB231 and BT549

Early in the study, MDA-MB231 and MCF7 cells were used as models as they represent a typical mesenchymal and epithelial-like breast cancer cell lines respectively (Bambang *et al.* 2009b, Zhang *et al.* 2010). Further investigations revealed that overexpression of ERp29 altered the morphology of MDA-MB231 but not MCF7 cells, which already exhibit epithelial appearance. Of note, MCF7 cells express higher level of ERp29 compared to MDA-MB231 cells (Bambang *et al.*, 2009b). In order to confirm and explore more of the finding, BT549 cell was chosen as it displays similar morphology as MDA-MB231 cell (*i.e.* fibroblast-like). BT549 is also an invasive breast cancer cell line that expresses comparable level of ERp29 as MDA-MB231 (Bambang *et al.*, 2009b). Surprisingly, while ERp29 overexpression induces parallel morphological changes in BT549 and MDA-MB231 cells, the molecular mechanisms are rather different.

Cell lines are widely used to investigate tumor formation and progression, indeed a considerable part of our knowledge on breast carcinomas is based on *in vivo* and *in vitro* studies performed with breast cancer cell lines. Based on the phenotype and invasiveness - thus supporting the idea of EMT in breast cancer progression - breast cancer cell lines can be classified into three groups (Lacroix *et al.*, 2004). The first includes luminal epithelial-like cells (*e.g.* MCF7) that express high level of epithelial markers, grow as cluster of polygonal cells with low invasive potential. The second group includes the weak luminal epithelial-like cells which express reduced amount of epithelial markers and grow in clusters of loosely attached cells. The last is

mesenhycmal-like group which expresses mesenchymal instead of epithelial markers, has fibroblastoid morphology, and is highly invasive. MDA-MB231 and BT549 cells are classified under the third group. In fact, microarray study showed that according to their gene expression pattern, both cell lines, as well as Hs578T, can be grouped together with primary breast stromal/fibroblast cell strain (HMS32) and an immortalised stromal cell line (UTSW) (Ross *et al.*, 2001). Due to their aggressiveness, MDA-MB231 and BT549 are capable of forming tumor *in vivo*, therefore often used as models in study of breast cancer formation and metastasis.

Despite the similarities, MDA-MB231 and BT549 cells exhibit many disparities. Firstly, BT549 cell line originates from primary papillary invasive ductal carcinoma while MDA-MB231 is derived from pleural effusion of invasive ductal carcinoma and has lost signatures of its origin tissue (Coutinho and Lasfargues, 1978 - unpublished data; Ross *et al.*, 2001). The source of these cell lines may reflect their EMT status; the primary-origin of BT549 cells indicates that they are undergoing EMT, in contrast MDA-MB231 cells which has broken away from starting tumor may have undergone complete EMT. Study on ovarian carcinoma has shown that EMT/MET modulators, such as E-cadherin, SNAI1, SNAI2, and ZEB2 are differentially expressed in effusions compared to primary tumors (Elloul *et al.*, 2010). This difference in extent of EMT may explain the observed molecular and biological variances in ERp29-induced MET between MDA-MB231 and BT549 cells.

Additionally, BT549 cells are known to express higher level of N-cadherin (a cadherin-switch component implicated in molecular/behavioral EMT/MET) compared

to MDA-MB231 cells which express, if at all, very low level of N-cadherin (Iwatsuki *et al.*, 2010). This discrepancy in N-cadherin expression may also explain different responds observed upon overexpression of ERp29. The expression of N-cadherin in epithelial-like PMC42-LA human breast cancer cells has been suggested to restrain EMT-like responses upon epidermal growth factor (EGF) induction (Ackland *et al.*, 2003).

4.2 Complete and incomplete MET induced by ERp29

Developmental EMT is a chronological process that follows strict series of events which are well defined and typically accompanied by cell fate decisions. On the other hand, pathological EMT associated with cancer typically lacks the coordinated and orderly induction of a complete EMT (reviewed in Gavert *et al.*, 2008). The highly variable environmental cues coupled with genetic heterogeneity of the cancer may lead to varying degrees of epithelial plasticity and reactivation of developmental migratory programs. Thus, while cancer-EMT always involves morphological changes, the epithelial and mesenchymal marker regulations as well as migration and invasion capacity may differ widely. This is further complicated by the use of *in vitro* system which does not always allow epithelial cells to achieve full polarity. Nevertheless, *in vitro* cell culture based studies should not be underestimated as their simplicity has allowed relatively easy identification of many key players and mechanisms important in EMT/MET, including β-catenin and SNAI2 (Conacci-Sorrell *et al.*, 2003).

In this study, overexpression of ERp29 is shown to induce complete MET in MDA-MB231 cells and incomplete MET in BT549 cells. These suggest that there may be two different signaling pathways regulated by ERp29 in a context-dependent manner; one is associated with genetic reprogramming which results in molecular/behavioral alterations while the other regulates actin cytoskeleton, tight junctions, and polarity proteins resulting in morphological changes. In many systems, the induction of EMT involves upregulation of transcription factors (such as Snail, ZEB, and bHLH families) which regulate the expression of epithelial and mesenchymal markers as well as MMPs linking the molecular and behavioral aspects of EMT. These transcription factors are also known to regulate expression of tight junction and polarity modules; Snail family downregulates occludin and claudins as well as members of Crumbs complex; ZEB family downregulates ZO proteins (reviewed in Xu *et al.*, 2009; Moreno-Bueno *et al.*, 2008). There was, however, no change in the expression of mRNA level of any of the tight junction and polarity modules examined (Figure 3-3 A), thus excluding the involvement of transcriptional regulation of these proteins in ERp29-induced MET.

Differential regulation of the morphological and molecular/behavioral aspects in EMT/MET has been observed in several studies. When epithelial nontransformed mouse mammary cell line NMuMG undergoes EMT upon addition of transforming growth factor-β (TGFβ), E-cadherin expression is not downregulated until day 3 of treatment, on the other hand the morphological change is evident from day 1 (Maeda *et al.*, 2005). Similar to present study, the change in cell shape is accompanied by

changes of actin cytoskeleton, ZO1 localization, as well as fibronectin induction, all of which occur separately (*i.e.* earlier) than changes in E-cadherin expression and/or localization. Induction of EMT in human mammary epithelial cells (HMECs) also shows that loss of ZO1 and gain of fibronectin are early events (hours after induction) while loss of E-cadherin is only evident when morphological alterations have taken place (3 days after induction) (Lindley *et al.*, 2010). It is interesting to note that in the two models, N-cadherin is differentially regulated. Compared to NMuMG/E9 cells where upregulation of N-cadherin is observed early, cadherin switch in HMECs is a late event. Unfortunately, lack of information on N-cadherin in present study prevents further investigation in regards to association of N-cadherin with morphologic and/or molecular MET.

The observations described above propose that loss of E-cadherin, and possibly transcriptional regulation of other markers as well, is independently regulated from morphological changes during EMT/MET. Indeed, the study on NMuMG cells revealed that while cadherin switching is essential for behavioral changes, it is not required for the morphological alterations that accompany EMT (Maeda *et al.*, 2005). When the upregulated N-cadherin in TGFβ-induced EMT is depleted by siRNA treatment, morphological change is still observed. In contrast, depletion of N-cadherin in these EMT cells results in slower migrating cells. The same phenomenon is also observed in immortalized breast cells, MCF10A (Maeda *et al.*, 2005).

In agreement with studies described earlier, present study suggests that overexpression of ERp29 independently modulates two pathways leading to induction

of MET. The morphologic MET is activated in both MDA-MB231 and BT549 cells while the molecular/behavioral MET is only active in MDA-MB231 cells.

4.3 Associations with TGFβ-induced EMT

The proposal that ERp29 regulates different downstream routes in the induction of MET is reminiscent of the activation of various pathways in growth factor-induced EMT, in particular TGFβ. TGFβ is a ubiquitously expressed cytokine that is closely associated with the carcinogenesis. Early in tumor progression, TGFβ acts as tumor suppressor by inducing apoptosis and cell cycle arrests. Once tumor cells acquire the resistance for the growth-inhibitory effect (*i.e.* in later stage of carcinogenesis), TGFβ may promote invasion and metastasis by inducing EMT (Oft *et al.*, 1996). In fact, TGFβ is the most potent and well studied amongst other EMT inducers. TGFβ signals through two transmembrane kinase receptors, transforming growth factor-β receptor I (TβRI) and transforming growth factor-β receptor II (TβRII) (Feng *et al.*, 2005). Ligand binding to TβRII promotes the recruitment and phosphorylation of TβRI which will phosphorylate downstream effectors. In general, there are two pathways through which TGFβ can induce EMT, the canonical Smad-dependent pathway and the non-canonical Smad-independent pathway.

In the canonical pathway, activated TβRI phosphorylates Smad2 and Smad3 which will form a complex with Smad4. Together they translocate into the nucleus where they interact with DNA binding partners and regulate expression of various genes, among which are the EMT inducing factors (Işeri *et al.*, 2011). Therefore, the

canonical pathway is often considered as gene expression/transcriptional-dependent pathway, contributing to the molecular changes in EMT. This pathway might be associated with ERp29-induced molecular/behavioral MET.

The non-canonical TGFβ signaling takes place through its ability to stimulate various alternative pathways such as small GTP-binding proteins (Ras, Rho, and Rac1); phosphoinositide-3- kinase (PI3K), AKT, and mTOR; MAP kinases (p38 MAPK, ERK1/2, and JNK); Par6; NF-κB and Cox-2 (reviewed in Taylor *et al.*, 2010). Of interest, TGFβ-induced EMT has also been shown to result in the downregulation of Par3 both at transcriptional and translational levels, leading to redistribution of Par6 and aPKC from cell membrane (Wang *et al.*, 2008). Since present study shows that overexpression of ERp29 does not alter mRNA level of Par3, this pathway will not be discussed further.

Two of the non-canonical pathways are of interest in this study as they regulate polarity and tight junction proteins as well as actin cytoskeleton organization, thus contribute to morphological aspect of EMT. The first involves polarity module Par6. Par6 has been demonstrated to play a role in TGFβ-induced EMT by mediating the dissolutions of tight junctions (Ozdamar *et al.*, 2005). Upon ligand binding, TβRII is recruited to tight junctions where TβRI and Par6 colocalize. This allows the phosphorylation of Par6 which leads to recruitment of Smurf1. Smurf1 promotes degradation of RhoA resulting in the dissolutions of tight junctions and therefore EMT. This pathway is independent of Smad as expression of non-phosphorylatable mutant version of Par6 does not block the Smad-mediated transcriptional activation of

vimentin gene expression (Ozdamar *et al.*, 2005). Involvement of polarity and tight junction proteins in this model is an attractive feature in describing ERp29-induced MET. The absence of changes in Par6 expression, however, seems to suggest otherwise. It is worth noting that present study does not provide the phosphorylation status of Par6 upon ERp29 overexpression, warranting further investigations to confirm the hypothesis.

In contrast to the Par6-mediated EMT that involves the degradation of RhoA, another non-canonical pathway results in activation of RhoA. The differential regulation of RhoA in TGFβ-induced EMT has been attributed to its downstream effectors. The capacity of RhoA to maintain tight junctions is believed to involve mDia, while ROCK activity leads to dissolution of cell-cell contacts and formation of stress fiber (Sahai *et al.*, 2002). In NMuMG cells, mink lung cell line (Mv1Lu), pancreatic tumor cell line (BxPc3), and primary mouse keratinocytes, EMT induced by TGFβ-treatment has been shown to require activation of RhoA (Bhowmick *et al.*, 2001). Blocking RhoA or ROCK activation inhibited stress fiber formation as well as changes in cell shape but did not affect E-cadherin integrity. Studies from renal proximal tubular cells support this notion. Activation of RhoA, not Smad, is responsible for the morphological change in TGFβ-induced EMT and inhibition of ROCK inhibits this effect (Tian *et al.*, 2003). It is interesting to note that in ERp29-induced MET, morphological change is accompanied by reduction of stress fiber formation which is supported by the decrease of phosphorylated form of MLC. As mentioned earlier, the phosphorylation of MLC is mediated by ROCK.

Activation of multiple downstream pathways in ERp29-induced MET is reminiscent of modulation of multiple effectors in TGFβ-induced EMT. Furthermore, many regulators that have been demonstrated to be important mediators in TGFβ-induced EMT (*i.e.* E-cadherin repressors and formation of stress fiber via pMLC) are shown to be differentially regulated upon overexpression of ERp29. Therefore, it is tempting to raise the possibility that ERp29 is involved in TGFβ-induced EMT, possibly inhibiting Smad canonical pathway and/or RhoA non-canonical pathway.

4.4 Restoration of apical-basal polarity

Present study shows that in inducing epithelial morphology, ERp29 restores apical-basal polarity in both MDA-MB231 and BT549 cells (Figure 3-3 and 3-4). Initiation of cell polarity is a complex process involving polarity modules and junctional complexes which physically separate the apical and basal domains. It is proposed that polarity and tight junction proteins as well as actin cytoskeleton work intimately together to establish final epithelial polarity (reviewed in Godde *et al.*, 2010). The junctional complexes play a complicated role in polarization. On one hand, their formation is believed to drive polarization, on the other hand the formation of mature apical junctional complexes is often used as a benchmark of completed polarization. Recently, two opinions have been suggested in describing the establishment of apical-basal polarity; the classical model in which cell adhesions initiates polarization and non-classical model where cell polarization occurs without cell-cell contacts (reviewed in Wang *et al.*, 2007).

Upon initiation of cell polarization cues (such as regulation of growth factors), cells rearrange their actin cytoskeleton which allows the apical distribution of Par complex protein, Par3. In the classical model, Par3 localization requires E-cadherin and is mediated by tight junction structural component junctional adhesion molecule-1 (JAM1) and the adherens junction component nectin at cell-cell contact sites (Ebnet *et al.*, 2001; Itoh *et al.*, 2001; Takekuni *et al.*, 2003). In contrast, initial cell-cell contacts have been shown not to be crucial, as activation of the polarity regulator LKB1 in single mammalian intestinal cells lacking junctions results in polarization; supporting non-classical model (Baas *et al.*, 2004). Furthermore, in some cases Crumbs and Par complexes have been suggested to initiate polarity without adhesion (Wang *et al.*, 2007). In either model, redistribution of Par3 is then followed by separation of other members of polarity complexes as well as transportation of (or the rest of) cell-cell junction proteins to cell periphery. The polarity proteins and junctional proteins will then depend on each other for their maturation. For example, adherens junction component is needed to maintain Par3 at tight junctions and in turn Par3 has been shown to be important in tight junction formation as its depletion disrupts the tight but not adherens junctions (Halbleib *et al.*, 2006). In addition, Par complex is established upon recruitment of Par6-aPKC complex by binding of Par6 to Par3 located at the tight junctions and downregulation of Par3 results in loss of cell polarity as it redistributes Par6-aPKC complex from cell membrane (Chen *et al.*, 2006).

Considering the importance of Par3 in the establishment of apical basal polarity, it may play similar role in ERp29-induced MET. This might also be true even in BT549 model where only a small portion of the upregulated Par3 is redistributed to cell periphery. Of note, small fraction of another polarity protein, PALS1, has been shown to be adequate for tight junction assembly (Straight *et al.*, 2004). Overexpression of ERp29 induces rearrangement of actin cytoskeleton allowing localization of Par3 to cell membrane. This promotes the assembly of ZO1 and Scribble at cell-cell contact sites which together lead to restoration of epithelial polarization and morphology. Further investigation is needed to learn if other polarity and/or cell-cell junction modulators are involved in ERp29-induced MET.

4.5 ERp29: functions in MET and secretion

Present study demonstrates that overexpression of ERp29 restores epithelial morphology in breast cancer cells. Epithelial cells play critical roles in many normal physiologies (Baum *et al.*, 2008; Yang *et al.*, 2008). They cover organs and body cavities thereby imparting mechanical protection, mediate absorption, sensory, and secretion. Epithelia function in wide variety of biological processes from nutrient absorption in the intestines to gaseous exchange in the lungs to lactogenesis in the mammary gland. The last example is of particular interest as ERp29 is also known to mediate secretion of various entities, including proteins in milk production (see Introduction).

In mammary epithelia, permeability of epithelia is closely related to milk secretion where the number of tight junction strands varies between pregnant and lactating stages (Morgan *et al.*, 1982). Decrease permeability during pregnancy is achieved through increasing the number, as well as organization, of tight junctions. This enhancement of tight junction barrier is thought to involve increased expression as well as phosphorylation of ZO1, which can be induced upon addition of prolactin or glucocorticoids (Singer *et al.*, 1994). Therefore, tight junction components, in particular ZO1, is crucial for optimal milk secretion. Considering that the role of ERp29 as putative secretory protein has also been implicated in milk production (Mkrtchian *et al.*, 2006), its ability in promoting MET (by inducing ZO1) might also contribute to the secretory function.

It is also worth noting that the polarized trafficking machinery is an adaptation of the secretory and endocytic systems. In MDCK cells apical and basolateral polarity proteins are synthesized at the endoplasmic reticulum, transported to the Golgi complex and sorted at the trans golgi network into distinct apical and basolateral vesicular routes (Simons *et al.*, 1990; Rodriguez-Boulan *et al.*, 1992). This, again, suggests that the role of ERp29 in inducing MET (by restoring polarity and tight junction modulators to their proper location) might be interlinked with its secretory function.

4.6 ERp29: friend or foe?

Association of ERp29 and cancer progression has been puzzling; studies have implied it as tumor suppressor as well as oncoprotein (Table 1-3). Current study has demonstrated the functional role of ERp29 in inducing MET and epithelial cell polarity, providing a novel insight into its tumor suppressive function in breast carcinogenesis. This notion, however, has to be taken with care as MET may be seen as both support or hindrance in metastasis.

EMT has been implicated as a mechanism that mediates the escape of cancer cells from primary tumors therefore facilitating metastasis. Thus, molecular intervention that can counteract this transition by inducing MET, including ERp29, might be considered as tumor suppressor. This is supported by the finding that level of ERp29 is downregulated in breast tumor samples compared to their normal counterparts and there exists a negative relationship between ERp29 and breast cancer stage/grade (Bambang *et al.*, 2009b). In addition, overexpression of ERp29 inhibits tumor growth *in vivo*. Similar observations are found with a known tumor suppressor EphB3 whose expression is significantly reduced in advanced Dukes' stage of colon cancer and overexpression induces MET in human colon cancer cells HT-29 (Chiu *et al.*, 2009).

On the other hand, MET has also been explored as an integral part of cancer progression by mediating the metastatic formation at secondary sites. Furthermore, overexpression of ERp29 has been shown to increase resistance to chemotherapeutic agent doxorobucin by attenuating its apoptotic effect (Zhang *et al.*, 2010a). Thus, the presence or elevated level of ERp29 might essentially confer worse prognosis. Indeed,

study in bladder carcinoma cells reveals that although generated from same genetic background, more metastatic cell sublines acquire epithelial characteristics through MET (Chaffer *et al.*, 2006). These epithelial-like sublines dramatically increased metastatic ability upon intracardiac or intratibial injection but not orthotopic inoculation, indicating the need of EMT for escape from primary tumor.

Armed with information at hand, precise role of ERp29 in breast carcinogenesis, in particular metastasis, remains unclear. Mechanistic and experimental model studies on the involvement of ERp29 in cancer cell metastasis are vital in solving this uncertainty. It should be noted that, like TGFβ, ERp29 may act as both tumor suppressor and promoter; its downregulation may promote tumor progression in primary tumors, while upregulation of ERp29 in aggressive tumor cells may involve MET and thus facilitates distant metastasis in the late stage. In all, it is imperative to elucidate the pathological functions of ERp29 in cancer progression and to investigate its clinical potential as a novel therapeutic target in metastatic cancer treatment.

4.7 Conclusions

Similar to other reticuloplasmins, ERp29 is involved in cancer progression. This study reveals that overexpression of ERp29 results in the induction of complete and incomplete MET in MDA-MB231 and BT549 breast cancer cells, respectively. These observations lead to the proposal of two potential mechanisms regulated by ERp29, leading to different aspects of MET (Figure 4-1).

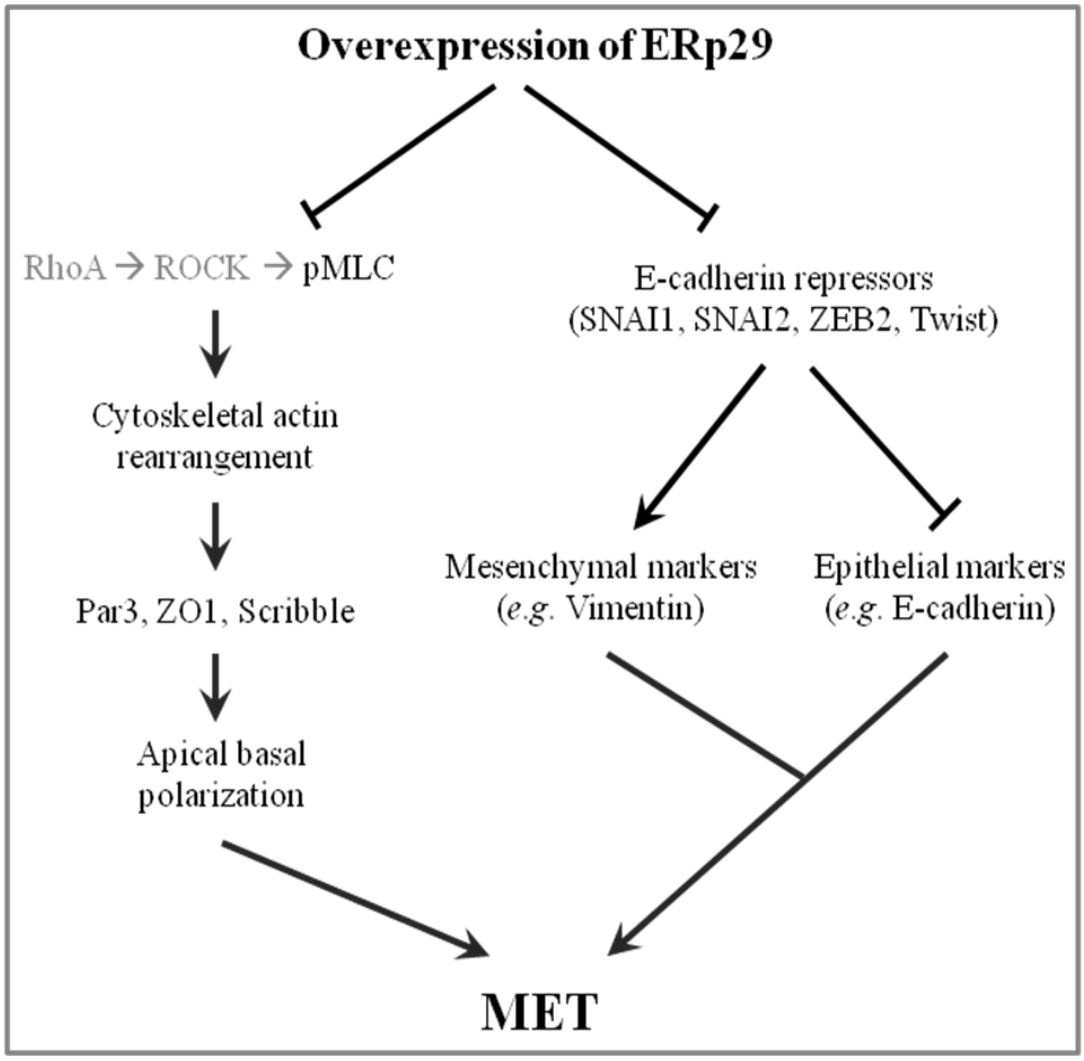


Figure 4-1 Proposed mechanism in ERp29-induced MET. Overexpression of ERp29 activates two downstream pathways. The first includes regulation of actin cytoskeleton (possibly through downregulation of RhoA and/or inactivation of ROCK), tight junctions and polarity complexes. The second includes downregulation of E-cadherin repressors and subsequent regulation of epithelial and mesenchymal markers.

4.8 Future works

The novel finding that ERp29 regulates MET provides the platform to better understand the role of ERp29 in breast cancer progression. However, it is not clear yet whether ERp29-induced MET inhibits metastasis by counteracting EMT or promotes metastasis as an independent mechanism. In previous study, ERp29 has been shown to inhibit primary tumor growth when ERp29-overexpressing MDA-MB231 cells are injected subcutaneously (Bambang *et al.*, 2009b). Unfortunately, investigation on metastasis was not performed. Varying the site of inoculation allows examination of different stages of metastatic process (Chaffer *et al.*, 2006). Metastatic growth observed following orthotopic injection demonstrates that cells are able to complete all metastatic stages including migration and invasion from primary tumor, intravasation to bloodstream, survival in the circulation, extravasation, as well as development of secondary tumor. Intercardiac injection reveals cells' ability to survive in circulation, extravasate, and form metastatic growth. Direct injection at secondary sites bypasses all but the last stage of metastasis, namely the secondary tumor formation. Studies performed under these settings will shed light on the implication of ERp29-induced MET.

It is proposed that regulation of RhoA and its effector ROCK might be the mechanism underlying ERp29-induced MET (Figure 4-1). To confirm this notion, examination of expression level, localization, as well as activation status of the above mentioned regulators is necessary. Furthermore, induction of MET by ERp29-overexpression has been associated with TGFβ-induced EMT. Therefore investigating the regulation of

TGFβ's receptor, such as TβRI and TβRII, may prove useful. In addition, it might also be interesting to investigate if ERp29 inhibits TGFβ-induced EMT. Of note, inhibitor of TGFβ-induced EMT such as glucocorticoid dexamethasone, has been shown to promote MET (Zhang *et al.*, 2010b).

This study shows that overexpression of ERp29 regulates MET in mesenchymal-like MDA-MB231 cells and basal-like BT549 cells by restoring epithelial characteristics. The importance of ERp29 in maintaining epithelial integrity may be explored by downregulating the protein in epithelial MCF7 breast cancer cells, which express high level of ERp29. Preliminary study reveals that ERp29 knockdown accelerates proliferation rate, increases mesenchymal markers and decreases epithelial markers, and increases migration and invasion capacity in MCF7 cells (Bambang *et al.*, 2009b).

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